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Regulation of pteridine biosynthesis

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INTRODUCTION

Pardee (81) suggests that a bacterium contains three groups of enzymes based upon the rate at which the substrate is used. The first group of enzymes catabolizes a primary carbon source at the rate of 10^8 molecules of substrate per minute per cell. The second group of enzymes synthesizes the major cell metabolites, amino acids, purines and pyrimidines, and uses 10^6 molecules of substrate per minute per cell. The last group of enzymes synthesizes the minor constituents, vitamins and coenzymes, and uses 10^3 molecules of substrate per minute per cell. Thus, the rate of vitamin synthesis is 1000 times less than the rate of amino acid synthesis (63).

The regulation of the enzymes in the first two groups is defined in terms of constitutivity (20, 45), induction-repression (21, 45) and negative feedback inhibition (53, 113, 131). The constitutive enzymes are produced at a relatively constant rate independent of nutritional conditions and the enzyme concentration varies no more than 5-fold (82). In contrast to the constitutive enzymes, the enzymes subject to induction-repression are synthesized at different rates depending upon the nutritional conditions. The rate of enzyme formation may vary by at least 10-fold (2, 9, 130) and some rates of enzyme formation can vary by a factor of 1000 or more (19, 38, 99). Finally, for enzymes subject to negative feedback inhibition, the activity of an enzyme can be decreased or inhibited by a product of the biosynthetic pathway in which the enzyme functions, thereby maintaining a constant intracellular level of product.

The regulation of the enzymes in the third group, those responsible

for the synthesis of the minor constituents, is poorly understood. Pardee and co-workers (81, 82, 125) state that there is no reason to believe that the control mechanisms for all enzymes are similiar and imply that an elaborate control mechanism for the minor pathways might not be economical. Therefore, the enzymes of the minor pathways are probably produced at a steady rate slightly greater than is essential for growth (constitutivity). Since several vitamin reactions are carried out at high velocities, nearly equal to the velocity of the glycolytic enzymes, the overall rate of vitamin synthesis is presumably limited by the number of enzymes, one or a few enzyme molecules per cell (63). Thus, the regulation of vitamin biosynthesis is closely linked to the regulation of enzyme synthesis. Repression is suggested as the mechanism which maintains the low levels of the flavin biosynthetic enzymes (95, 125). However, only nicotinic acid mononucleotide pyrophosphorylase (38) and the enzyme which synthesizes biotin from desthiobiotin (78, 79, 80) are known to be subject to induction-repression. It is questioned whether negative feedback inhibition plays a significant role in the regulation of vitamin biosynthesis (81, 82, 125). There have been direct but unsuccessful searches for negative feedback inhibition using the known enzymes responsible for pyridine nucleotide coenzyme biosynthesis (38) and the initial enzyme in pteridine biosynthesis, GTP-cyclohydrolase (18, T. Shiota, University of Alabama Medical Center, Birmingham, personal communication). The only evidence for negative feedback inhibition is the strong inhibition of dihydroneopterin aldolase, an enzyme in the folic acid pathway, by its immediate product, 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine, and weak inhibition by dihydropteroic and dihydrofolic acids (62). It is probable that other vitamin

biosynthetic enzymes are also subject to feedback inhibition, but since so few vitamin enzymes are known, either the wrong enzymes or the wrong affectors have been used in attempts to demonstrate feedback inhibition.

There is so little known about the biosynthetic pathways of the minor metabolites that it is not too surprising that the regulation of these pathways is not understood. The terminal enzymatic reactions are known for most vitamin pathways, but the substrate and the initial enzymatic reactions are unknown for all but the folic acid and possibly the riboflavin pathways. The following are some reasons why so little is known about the biosynthesis of the minor metabolites: the vitamins are synthesized at a low rate, about 1 nmole per mg dry weight of organisms per hour compared to 1 to 3 μmoles for the amino acids (63, 64, 65, 66, 67); the substrates are unknown; the intermediates are unknown; the intermediates are synthesized in extremely small quantity; the intermediates are usually transitory and the suspected intermediates are not commercially available.

The folic acid pathway is the best defined pathway of the minor metabolites. Even so, only three enzymes of the folate pathway are known. The enzymes are large complexes (18, 62) and two are known to catalyze more than one reaction. Still, no mechanism has been proposed for the regulation of the folate pathway.

It has been shown (108) that GTP-cyclohydrolase is the first enzyme in the biosynthesis of pteridines and folic acid-like compounds in *Staphylococcus epidermidis*. The initial reaction opens the imidazole ring of guanosine-5'-triphosphate (GTP) and eliminates carbon atom-8 as formic acid. A similar purine ring-opening mechanism has been reported in the biosynthesis of riboflavin (35, 68, 70), toxiflavin (56, 57, 58),

pyrrolopyrimidine nucleosides (112) and unconjugated pteridines (33), as well as folic acid-like compounds (17, 18, 102, 108). When more than one of these biosynthetic pathways is present in an organism, there must be more than one mechanism to remove C-8 of purines or a branched biosynthetic pathway with a common precursor and probably some common intermediates. Since both riboflavin and folic acid-like compounds are synthesized by *S. epidermidis*, it is possible that the two pathways share a common precursor and the two pathways have some common intermediate compounds. If this is true, then the regulation of the early steps of folic acid biosynthesis can not be adequately described without also describing the regulation of riboflavin biosynthesis.

At the initiation of this study, the knowledge of folate biosynthesis in *S. epidermidis* was limited to the biosynthetic pathway and the types of natural products synthesized (10, 40, 71, 108, 123, 124). Very little was known about when or how much vitamin was synthesized or if the quantity varied with physiological changes. Essentially nothing was known about riboflavin biosynthesis in *S. epidermidis*.

The approach that I have used to examine the regulation of pteridine biosynthesis is: first, to examine how folic acid and riboflavin biosyntheses change in response to the physiological changes that occur during growth and in different culture media; second, to examine the variation in specific activity of enzymes from the pteridine pathway, the folic acid pathway and the riboflavin pathway in response to physiological changes; and third, to provide evidence that the folic acid and the riboflavin pathways are related.

LITERATURE REVIEW

The isolation and identification (29, 55), chemistry (83) and biology (116) of pteridines have been reviewed. Reviews of the pteridine cofactors have been restricted largely to folic acid (43, 87, 109), but recently the unconjugated pteridine cofactors have been emphasized (28, 47).

The pteridine ring system consists of fused pyrimidine and pyrazine rings (pyrimido-[4,5-b]-pyrazine). The naturally-occurring pteridines have an amino and a hydroxy substitution on the 2 and 4 positions respectively. The 2-amino-4-hydroxypteridine derivative has been assigned the trival name of 'pterin' and the 2,4-dihydroxypteridine derivative has been assigned the trival name of 'lumazine'. Of the nine possible tautomeric forms for pterins, only 2-amino-3,4-dihydro-4-ketopteridine predominates in aqueous solution (14, 84).

The pteridines have been subdivided into conjugated and unconjugated classes. The conjugated pteridines are the pteroic acid-like and the folic acid-like compounds. All other pteridines are classed as unconjugated pteridines (47).

Purine precursors of pteridines and riboflavin

Because of the structural resemblance and the incorporation of radioactive formate and glycine into analogous portions of purines, pteridines and the isoalloxizine ring of riboflavin (11, 16, 31, 85, 86), a biological precursor-product relationship was suspected for some time.

Weygand and Waldschmidt (121) provided evidence that the biosynthetic pathways of purines and pteridines were closely related. Since then, evidence has accumulated that shows purines can be precursors of pteridines

(1, 11, 12, 115, 122, 132). Vieira and Shaw (115) established that radioactivity from adenine-2-^{14}C but not adenine-8-^{14}C was incorporated into pteroyltriglutamic acid (teropterin) in whole cells of *Corynebacterium* sp.

Reynolds and Brown (92, 93) demonstrated in cell-free extracts of *Escherichia coli* that pteridines were synthesized from guanosine, guanosine nucleotides or guanine and 5-phosphoribosyl-1-pyrophosphate. No other purine compounds formed pteridines which would react with *p*-aminobenzoylglutamate to yield folic acid. Guanosine-5'-triphosphate (GTP) was shown to be a precursor of pteridines in cell-free extracts of *Comamonas* sp. (23), *E. coli* (18, 41, 42), *Lactobacillus plantarum* (102), *Pseudomonas cocovenans* (56, 57, 58), *Pseudomonas* sp. (33), *Salmonella typhimurium* (24) and *S. epidermidis* (108).

Adenine, guanine, xanthine and hypoxanthine were shown to stimulate riboflavin production in cultures of *Eremothecium ashbyii* (60) and in *Saccharomyces cerevisiae* (30). McNutt (68, 69) demonstrated in cultures of *E. ashbyii* that radioactive adenine was incorporated into riboflavin. Furthermore, the nucleic acid bases stimulated riboflavin synthesis to a greater extent than the nucleoside or nucleotide with the exception of guanine-5'-monophosphate (GMP). By using a double labeled purine, xanthine-^{14}C-^{15}N, McNutt (70) unequivocally demonstrated the direct incorporation of the diamino-pyrimidine moiety remaining after the loss of C-8 into riboflavin. Bacher and Lingens (4) used a polyauxotrophic mutant of *Aerobacter aerogenes* with a genetic block in the purine pathway prior to inosine-5'-monophosphate (IMP) and a genetic block between xanthosine-5'-monophosphate (XMP) and GMP. Xanthine-2-^{14}C was incorporated into xanthosine but not riboflavin while guanine-2-^{14}C was incorporated into riboflavin without

significant dilution. Both adenosine-5'-monophosphate (AMP) and IMP were excluded as precursors of riboflavin since there was no significant isotope dilution in the presence of unlabeled adenine or hypoxanthine. They concluded that a guanine derivative was the precursor of riboflavin. Baugh and Krumdieck (7) reached the same conclusion using decoyinine which blocks the conversion of XMP to GMP and a mutant of *Corynebacterium* sp. which could not convert either xanthine or guanine to adenine. Guanine was also shown to serve as a precursor of riboflavin in *E. ashbyii* (35) and *E. coli* (37). Since all of the precursor studies used whole cells, it was impossible to determine if guanine or a nucleoside or nucleotide derivative of guanine was the true precursor.

Pteridine biosynthesis

The initial reactions in pteridine biosynthesis which results in the release of C-8 of GTP as formic acid have been well documented (17, 18, 23, 33, 56, 57, 58, 102, 108). The enzyme which catalyzes these reactions was named GTP-cyclohydrolase (18) and was shown to have a molecular weight in excess of 200,000 (18, T.Shiota, University of Alabama Medical Center, Birmingham, personal communication). Burg and Brown (18) used a highly purified preparation of GTP-cyclohydrolase from *E. coli* to show that the other product of the purine ring-opening reaction was a triphosphate ester of 2-amino-4-hydroxy-6-(D-*erythro*-1',2',3'-trihydroxypropyl)-7,8-dihydroneopterin (dihydroneopterin triphosphate). But, Cone and Guroff (23) reported that in cell-free extracts of *Comamonas* sp. the product was a cyclic phosphate ester of dihydroneopterin.

The ribose moiety of GTP was shown to be incorporated into pteridines

(52, 93) with carbon atoms 6, 7 and 9 of the pteridine originating from carbon atoms 2', 1' and 3' of ribose. Therefore, it was presumed that the ribose moiety of GTP was isomerized by an Amadori rearrangement. The Amadori rearrangement isomerizes an amino sugar to a 1-amino-1-deoxy-2-keto derivative of the sugar (36). GTP-cyclohydrolase was indirectly shown to catalyze the Amadori rearrangement of the ribose moiety by using 7-methyl-GTP, an analogue of GTP (129). The methyl group on N-7 of GTP does not inhibit the purine ring-opening reaction but does prevent the pteridine ring closure. The Amadori rearrangement product was identified by the ease with which it reacted with phenylhydrazine to give a ribose phenylosazone. It is interesting that C-8 of 7-methyl-GTP was not released as formic acid, suggesting that the Amadori rearrangement occurred after the purine ring cleavage but before the elimination of C-8 as formic acid. The Amadori rearrangement product is presumably a transitory intermediate which reacts immediately to yield dihydroneopterin triphosphate. A chemical model system indicated that the pteridine ring closure was nonenzymatic (110).

No biological intermediates between GTP and neopterin have been directly isolated or identified. The hypothetical intermediates should be derivatives of 2,4,5-triamino-6-hydroxypyrimidine with a phosphate ester of either ribose or a 1-deoxy-2-keto pentose unit substituted onto N-4. Some intermediates may also have a formyl group substituted onto the N-5 position. 2-Amino-4-(5'-triphosphoribosyl)amino-5-formamido-6-hydroxy-pyrimidine was chemically prepared from GTP and implicated as an intermediate in pteridine synthesis since it acted as a substrate for the enzymatic release of formic acid and the synthesis of dihydrofolate in extracts of *L. plantarum* (103, 107). The 4,5-diaminopyrimidine derivatives were

further implicated as intermediates when Levenberg and Kaczmarek (57) condensed the resulting 4,5-diaminopyrimidines, enzymatically derived from GTP, with glyoxal and identified the pteridine derivatives which were formed. Both pterin and lumazine derivatives were found which indicated that the 2-amino substituent of GTP must have been oxidatively deaminated.

The GTP-cyclohydrolase molecule is large and seems to catalyze the following reactions in the synthesis of dihydroneopterin triphosphate: two hydrolytic reactions in the release of C-8 of GTP as formic acid; the isomerization of the ribose moiety to a 1-deoxy-2-keto pentose unit; and possibly the oxidative deamination of the pyrimidine intermediates.

Jones and Brown (41, 42) demonstrated that dihydroneopterin was enzymatically converted to 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine (hydroxymethyldihydropteridine) with the loss of a two-carbon unit. Mathis and Brown (62) used a purified enzyme from *E. coli* and identified the two-carbon compound as glycoaldehyde. The enzyme, dihydroneopterin aldolase, was shown to have a molecular weight of 100,000 and to be heat stable. The enzyme was shown to be specific for dihydroneopterin. Neopterin, tetrahydroneopterin or phosphate esters of dihydroneopterin could not be used as substrate for the enzyme. The enzyme was strongly inhibited by the product of the reaction, hydroxymethyldihydropteridine, and weakly inhibited by dihydropteroic acid and dihydrofolic acid.

Pteroic acid and folic acid biosynthesis

Pteroic acid-like and folic acid-like compounds are synthesized by enzymatically coupling a pteridine by a methylene bridge at C-6 with the amino group of either *p*-aminobenzoic acid (pABA) or *p*-aminobenzoylglutamic

acid (pABG) (10, 15, 40, 100, 101, 104, 105, 106, 120, 123, 124). The following pteridine compounds in the presence of adenosine-5'-triphosphate (ATP) can serve as substrate for the biosynthesis of conjugated pteridines: 2-amino-4-hydroxy-6-pteridine aldehyde (15, 54, 100); 2-amino-4,6-dihydroxypteridine (46, 50); 2-amino-4-hydroxy-6-hydroxymethylpteridine (15, 100); 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine (10, 15, 39, 40, 71, 101); the monophosphate ester of hydroxymethyldihydropteridine (101, 104); and dihydroneopterin (41, 42, 73). In the absence of ATP, the pyrophosphate ester of hydroxymethyldihydropteridine (40, 77, 105, 106, 120) and dihydroneopterin triphosphate (18) were shown to be substrates in the synthesis of conjugated pteridines. Of the substrates examined in various bacterial extracts, hydroxymethyldihydropteridine pyrophosphate in the absence of ATP was shown to be the most active. The product of the reaction is either dihydropteroic acid (77, 105, 106, 120) or dihydropteroic phosphate (10, 40).

The enzyme which catalyzes the coupling reaction, dihydropteroate synthetase, apparently has little specificity for substrate or can catalyze several reactions. It has been shown that dihydropteroate synthetase can add a pyrophosphate moiety to hydroxymethyldihydropteridine to form hydroxymethylpteridine pyrophosphate (40, 72) as well as to couple pABA with the pteridine substrate. Then in the presence of ATP, Mg^{++} and glutamate, dihydropteroic acid can be enzymatically converted to dihydrofolic acid by extracts of *E. coli* (32). In contrast, dihydrofolic acid has not been reported as a natural product in *S. epidermidis*. Instead, the products in *S. epidermidis* seem to be pteroic acid-like compounds (10, 40, 71, 123, 124).

Riboflavin biosynthesis

Guanine or a guanine nucleoside- or nucleotide-derivative has been established as the purine precursor for riboflavin biosynthesis (4, 7, 35, 37). McNutt (69, 70) demonstrated that the four nitrogen atoms and C-4 of purines but not C-8 were incorporated into riboflavin by cultures of *E. ashbyii*. Hayes and Greenberg (35) used guanine-8-^{14}C and showed that C-8 of guanine was removed as a one carbon unit in cultures of *E. ashbyii* They reported that 70% of the radioactivity from C-8 of guanine was found as free formic acid, 20% was found in protein, 10% was incorporated into riboflavin and traces of radioactivity were found as free acetic acid. The radioactivity from guanine-2-^{14}C was incorporated into riboflavin.

The mechanism by which purines are converted to riboflavin has not been shown, but the 4,5-diaminopyrimidine compounds were implicated as intermediates since 6,7-dimethyl-8-(1'-D-ribityl)lumazine (dimethylribityl-lumazine), a known precursor of riboflavin (34, 88, 94, 117, 126), was chemically synthesized by a condensation of diacetyl with 4-(1'-D-ribityl-amino)-5-amino-2,6-dihydroxypyrimidine (61, 88, 89, 117). Both 2,5-diamino-6-hydroxy-4-(1'-D-ribitylamino)pyrimidine (3, 5, 6, 76) and 5-amino-2,6-dihydroxy-4-(1'-D-ribitylamino)pyrimidine (76, 98) were accumulated by riboflavinless mutants of *Saccharomyces cerevisiae* (3, 5, 6, 76) and *Aspergillus nidulans* (98) which further implicated the 4,5-diaminopyrimidine compounds as intermediates in riboflavin biosynthesis. Although these compounds were claimed to have been identified, only Bacher and Lingens (5) and Sadique *et al.* (98) provided any experimental data to characterize the nature of the compounds that accumulated. Neither the origin of the ribityl moiety nor the identification of the four-carbon compound which

condenses with the pyrimidine to form dimethylribityllumazine are known.

Riboflavin synthetase, the terminal enzyme in the riboflavin pathway, was characterized from extracts of *Ashbya gossypii* and bakers yeast (88). Winestock *et al.* (126) established that dimethylribityllumazine was the substrate for the enzyme riboflavin synthetase. Plaut (88) clearly established that two molecules of dimethylribityllumazine were consumed yielding one molecule of riboflavin. Plaut proposed that a four-carbon unit from one molecule of dimethylribityllumazine was donated to another molecule of dimethylribityllumazine to make riboflavin. The other product of this reaction was identified as 4-(1'-D-ribitylamino)-5-amino-2,6-dihydroxypyrimidine (117). Rowan and Wood (94) proposed a nucleophillic ring-opening of the dimethylribityllumazine followed by an aldol condensation as the mechanism by which a four-carbon unit is transfered from one molecule of dimethylribityllumazine to another.

Although the enzymology of riboflavin biosynthesis is still unclear, there is a considerable amount of information about flavin synthesis in growing cultures. Flavins were reported to be synthesized at a constant rate in excess of that required for growth in cultures of *E. coli* (125), *A. gossypii* (44) and *Candida guilliermondii* (95). There was no correlation between flavin synthesis and the supplimentation of the culture medium with a substrate that could be oxidized by flavoproteins (95). However, an iron-deficient medium inhibits growth but increases the rate of flavin synthesis (97). The rate of flavin synthesis was then inhibited by the addition of iron to the medium without any active multiplication of the cells. Kaplan and Demain (44) reported that growth temperature was important to riboflavin biosynthesis since *A. gossypii* synthesizes large

quantities of riboflavin at 28 C but a temperature of 37 C was inhibitory to flavin synthesis even though growth was not inhibited. Riboflavin synthesis can be stimulated by several compounds that presumably are not direct precursors of riboflavin. Glycine, tyrosine, hypoxanthine and Tween 80 are reported to stimulate flavin synthesis (44), but flavin synthesis ceases when there is a nitrogen deficiency (97). Uracil was shown to strongly inhibit riboflavin production in *E. ashbyii* (60), but in *S. cerevisiae* (30) uracil was shown to stimulate riboflavin synthesis. There is some confusion as to when flavins are synthesized. In *E. coli* (125) and *C. guilliermondii* (95) riboflavin is synthesized at a constant rate during exponential growth and ceases in the stationary phase of growth. But, it was shown that *A. gossypii* (44) initiates riboflavin synthesis in the decelerated phase of growth and continues to synthesize riboflavin through the stationary phase of growth.

Dynamic control

Bacteria are thought to respond to environmental stresses by altering their metabolic patterns in order to grow as rapidly as possible in their environment (81). Dynamic control, induction-repression (21, 45) and negative feedback inhibition (53, 113, 131) maintain the balance of intermediary metabolism within the organism. McIlwain (63) revaluated published data on vitamin synthesis and showed that the vitamins were synthesized at a rate about 1000 times less than the rate at which amino acids were synthesized. His report also showed that the vitamins were overproduced in all the bacterial systems that he evaluated.

Repression of the minor pathways

McIlwain (65, 67) reported that pantothenate was synthesized at a rate from 0.7 to 1.6 nmoles per mg dry weight of organisms per hour depending upon the culture medium. He reported that pantothenate biosynthesis starts at once without lag with a higher rate of synthesis during the production of the first few nmoles of pantothenate by the youngest cells. The rate of synthesis changes during different phases of growth as well as during growth in different culture media. Lacelles and Woods (54) reported that when *Bacterium coli* and *Staphylococcus aureus* were grown in the presence of sulfonamides and when *p*-aminobenzoateless mutants were grown on limiting amounts of pABA, the organisms became deficient in folic acid. But, the resting cell suspensions had an increased ability to synthesize folic acid-like compounds from pABA. Wilson and Pardee (125) reported that resting cell suspensions of *E. coli* synthesized flavins at a slower rate if the organism had been previously grown in a minimal medium instead of a rich medium. Collectively, this has been taken as indirect evidence for an induction-repression mechanism for the regulation of the enzymes in the minor pathways.

Pai and Lichstein (78) examined the effect of exogenous biotin upon biotin synthesis in cultures of *E. coli* when enzyme synthesis was inhibited by chloramphenicol or ultraviolet irradiation. They found that when cells were grown in the presence of biotin and transferred to a medium with chloramphenicol and lacking biotin, then the organism couldn't synthesize biotin. However, when cells were grown in the absence of biotin and transferred into a medium with chloramphenicol, then the cells synthesized biotin in the presence or absence of biotin. They concluded that this evidence

suggested an induction-repression mechanism and ruled out negative feedback inhibition. By examining the enzyme system which catalyzed the conversion of (±)-desthiobiotin to (+)-biotin in resting cell suspensions of *E. coli* and a biotinless mutant of *E. coli*, it was shown that the enzyme activity was absent in cells grown in the presence of (+)-biotin (79). The enzyme system was shown to be rapidly formed in a basal medium during early exponential phase of growth and repressed during the later phases of growth (80). Complete repression occurred only when the biotin content of the medium was raised above the intracellular concentration that was found in cultures upon the cessation of growth (80).

MacCormick *et al.* (59) showed that the level of pyridoxal phosphokinase in a thiamineless mutant varied with the concentration of thiamine in the growth medium. Imsande and Pardee (38) showed that the specific activity of the first enzyme of the pathway from nicotinic acid to nicotinamide adenine dinucleotide varied at least 200-fold depending upon the growth conditions. The enzyme was not subject to feedback inhibition.

Feedback inhibition of the minor pathways

Negative feedback inhibition was indirectly demonstrated by the use of whole cells and medium supplemented with large quantities of the vitamin (78, 125). Biotin biosynthesis in *E. coli* was not subject to feedback inhibition. *E. coli* (125) and *A. gossypii* (61) were reported to be impermeable to added flavins; therefore, it was impossible to determine the effects of flavins upon the enzyme activity in whole cells.

Mathis and Brown (62) presented evidence that dihydroneopterin aldolase, an enzyme of the folate pathway, is subject to competitive

inhibition by hydroxymethyldihydropteridine, one product of the enzymatic reaction. The other product, glycolaldehyde, had no effect upon the enzyme. 6-Formyldihydropteridine was also shown to be a potent inhibitor of the reaction. At very high concentrations, dihydropteroate and dihydrofolate also caused about a 30% inhibition of the enzyme.

Constitutivity of the minor pathways

Flavins were reported to be synthesized at a constant rate in excess of that required for growth in *E. coli* (125), *A. gossypii* (44) and *C. guilliermodii* (95, 96, 97). The folic acid-like compounds were also shown to be synthesized in excess and apparently at a constant rate (54, 75). It was suggested that excretion of an end product of a biosynthetic pathway indicates a control mechanism too weak to prevent oversynthesis (27, 74, 125). Wilson and Pardee (125) reported that the ratio of excreted flavins to intracellular flavins was between 0.8 and 8 during exponential growth under different growth conditions compared to about 0.01 for many amino acids. In addition, it was shown that vitamin biosynthesis continues in the absence of growth (54, 65, 75, 125). Since vitamin biosynthesis is not precisely adjusted to physiological needs, Pardee and Beckwith (82) suggested that a steady production of vitamin enzymes at a rate slightly greater than is essential for growth (constitutivity) is more economical than an elaborate control mechanism and regulated amounts of an enzyme.

EXPERIMENTAL PROCEDURE

Staphylococcus epidermidis, herein designated as strain W, was isolated and characterized by Koft and co-workers (10, 71, 123, 124). The organism was selected for its ability to excrete copious quantities of folic acid-like compounds (see Appendix Table 1 for the morphological and biochemical characteristics). Stock cultures of the organism were maintained on Trypticase Soy Agar (TSA) slants at 4 C and were transferred at 3-month intervals.

Synthetic medium

The synthetic medium was derived from the formula for Difco Folic Acid Assay Medium (FAAM) (26) by omitting sodium citrate, adenine, guanine, uracil and all vitamins except niacin. The final composition of the synthetic medium was: glucose, 10 g; vitamin free casamino acids, 6 g; *l*-cystine, 0.1 g; *l*-tryptophan, 0.1 g; nicotinic acid, 0.001 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $MnSO_4$, 0.01 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; K_2HPO_4, 1.2 g; KH_2PO_4, 0.8 g; and deionized water, 1000 ml. The medium was adjusted to pH 7.4 and then was autoclaved for 12 min at 121 C.

Pyruvate medium

A stock solution of amino acids was prepared and contained: *l*-glutamic acid, 1000 mg; *l*-aspartic acid, 900 mg; *l*-proline, 800 mg; *l*-tryptophan, 100 mg; *l*-valine, 800 mg; *l*-leucine, 900 mg; *l*-isoleucine, 300 mg; *l*-lysine, 500 mg; *l*-threonine, 300 mg; *l*-arginine, 500 mg; and deionized water, 1000 ml. The stock solution of amino acids was autoclaved for 15 min at 121 C and stored at 4 C. The *l*-cystine, 200 mg, was dissolved in

100 ml of deionized water to which 0.83 ml of concentrated HCl had been added, autoclaved and stored at 4 C. The l-cystine solution was added separately to the final medium. The final medium contained: sodium pyruvate, 5 g; amino acid solution, 100 ml; cystine solution, 10 ml; nicotinic acid, 0.001 g; thiamine, 0.001 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $MnSO_4$, 0.01 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; K_2HPO_4, 7 g; KH_2PO_4, 3 g; and deionized water, 890 ml. The complete medium was adjusted to pH 7.4 and then was autoclaved for 12 min at 121 C.

Growth in defined medium

To adapt *S. epidermidis* to grow in a defined medium, a heavy inoculum from a 24-hr TSA slant was added to 100 ml of either the FAAM, the pyruvate medium or the synthetic medium in a 250 ml Erlenmeyer flask and incubated for 12 hr at 25 C. Then the flask was incubated for an additional 18 hr at 35 C. There was no agitation of the culture medium during this initial 30-hr incubation period. A subculture using a 10% inoculum was made into a second flask of the defined medium. The second flask remained stationary for 3 hr and then was shaken on a gyrotory shaker (New Brunswick Scientific) for an additional 12 hr. The second flask was used as an inoculum for the growth studies. Both a temperature shift and low O_2 tension are essential in order to get *S. epidermidis* to grow in serial transfer in any of the defined media used. There is very poor growth (about 5×10^7 bacteria/ml) in the initial subculture into a defined medium, but growth is excellent (about 3×10^9 bacteria/ml) with subsequent subcultures in the defined medium.

Growth determinations

S. epidermidis was grown on a gyrotory shaker at 35 C in 1.5 l of medium in a Fernbach flask. At intervals during growth a 110 ml sample was removed. The optical density was read directly on a Spectronic 20 colorimeter (Bausch and Lomb) at 520 nm for the synthetic and pyruvate media and at 540 nm for the FAAM. Then the sample was diluted with sterile growth medium until a reading between 0.15 and 0.20 could be obtained. The observed reading was multiplied by the dilution factor to give a value called the corrected optical density (OD_c). The cell arrangement was examined under an oil immersion objective on a bright field microscope and then the number of organisms were determined with viable plate counts on TSA spread plates. Finally, the cells from the remaining 100 ml of culture medium were collected on a pre-washed, pre-weighed Millipore filter, 47 mm diameter and 0.22 micron pore size (Millipore Filter Corporation). The cells were washed with 25 ml of water and dried in a vacuum oven at 65 C until they reached a constant weight.

The corrected optical density is proportional to mass (49). The slope is different for each medium since there is a difference in both cell size and cell arrangement of *S. epidermidis* depending upon the growth conditions (see Appendix Fig. 1). Dry weight estimates can be made directly from a corrected optical density value with the aid of a standard curve of OD_c plotted against mass in a given medium. Thus, OD_c and dry weight will be used interchangeably as a measure of growth.

Viable plate counts of *S. epidermidis* are an inadequate measure of growth because the cell arrangement varies from predominately single organisms to pairs, tetrads, and clusters depending upon the phase of

growth and the growth medium.

Extraction of intracellular folate and riboflavin

Cells from 100 ml of culture medium were centrifuged at 29,000 X G for 5 min and washed once with 10 ml of deionized water. The cell pellet was resuspended in 5 ml of a 0.01% solution of Tween 80 and put into a screw cap tube. Toluene was added (1:10, v/v) to the cell suspension and tube was sealed and wrapped with aluminum foil to keep out light. The cell suspension was shaken on a Burrell wrist action shaker for 15 min at room temperature, then placed into a boiling salt brine (105 C) for 15 min, removed and shaken for an additional 10 min. The cap was removed, the toluene was driven off by boiling and the cell debris was removed by centrifugation at 29,000 X G for 20 min. The supernatant was collected, the volume was adjusted to 5.0 ml with deionized water and then the supernatant was examined for the quantity of folate and riboflavin extracted from the cells.

Paper chromatography

Samples (0.001, 0.01, and 0.1 ml) were spotted onto Whatman No. 3MM paper and were developed to a height of 25 to 30 cm by ascending chromatography in Solvent A (0.1 M potassium phosphate buffer, pH 7.0), Solvent B (the organic phase of n-butanol:glacial acetic acid:water, 4:1:5) or Solvent C (n-propanol:1% NH_4OH, 2:1) (see Appendix Table 2 for R_f values of standard pteridine, folate and riboflavin compounds).

The chromatograms were examined under ultraviolet light (254 nm) to detect absorbance or fluorescence. The folic acid-like compounds and the riboflavin derivatives were qualitatively detected by the technique of

bioautography (see Bioautography).

To detect radioactive compounds separated by paper chromatography, the developed and dried chromatogram was marked into 1-cm increments from the origin to the solvent front and in 3-cm widths (25). The 1 X 3 cm strips were removed and inserted into individual counting vials filled with 10 ml of scintillation cocktail (see Detection of radioactivity). The counting efficiency was determined by the channel ratio method and ranged from 89% to 94%.

Biological assay organisms

Streptococcus faecium (ATCC #8043, *Streptococcus faecalis* R) was used for the biological assay of pteroic acid-like and folic acid-like compounds in Folic Acid Assay Medium (Difco). *Lactobacillus casei* (ATCC #7469) was used for the biological assay for either pteroyl-γ-polyglutamate in Folic Acid Assay PGA Broth (BBL) or riboflavin compounds in Riboflavin Assay Medium (Difco) (see Appendix Table 2 for the type of compounds to which each organism will respond). Both organisms were maintained in stab cultures of Micro Inoculum agar and subcultured into 5 ml of Micro Inoculum Broth (MIB). The cells were washed three times in sterile assay medium prior to use as an inoculum for the biological assays. For use in the tube assays, *S. faecalis* was diluted 1:100 in sterile FAAM and *L. casei* was diluted 1:50 in the appropriate sterile assay medium for the determination of either pteroyl-γ-polyglutamate or flavins. For bioautography, the entire washed cell suspension from 5 ml of MIB was added to the appropriate assay medium.

Biotube assay

The quantities of flavins and folic acid-like compounds were determined by a modification of the tube assay procedures as described in the Difco manual (26). To determine the quantity of pteroate and folate compounds, the material to be examined was diluted, then known volumes of the diluted material were added to tubes containing 2.5 ml of double strength FAAM and the final volume was brought to 5 ml with water. The tubes were stoppered, autoclaved 12 min at 121 C, cooled, inoculated with 0.1 ml of washed and diluted (1:100) cells of *S. faecalis* and incubated at 35 C for 18 hr. The growth response was determined turibidimetrically with a Spectronic 20 colorimeter at 540 nm.

To determine the quantity of folic acid and pteroyl-γ-polyglutamate, the procedure was the same as for the pteroate and folate compounds except that Folic Acid Assay PGA Broth (BBL) was used with diluted (1:50) *L. casei* as the assay organism.

To determine the quantity of flavins, the tubes were prepared as for pteroates and folates except Riboflavin Assay Medium was used with *L. casei* as the assay organism. The incubation time was extended to 36 hr before the growth response was determined.

For each assay, a standard curve of growth response plotted against the concentration of either folic acid (0.05 to 0.5 ng/ml) or riboflavin (0.5 to 25 ng/ml) was determined. The quantities of biologically active compounds are referred to as 'folate equivalents' or 'riboflavin equivalents' depending upon the specific assay organism and assay medium.

Bioautography

The method of tetrazolium bioautography as reported by Usdin, *et al.*

(114) was modified to detect either folic acid-like compounds or flavins which have been separated by paper chromatography. To detect folic acid-like compounds, a base layer of 300 ml of FAAM with 2% agar was inoculated with washed cells of *S. faecalis* from a 5 ml MIB subculture and poured into a baking dish (22 X 34 cm). After the base layer solidified, a developed, dried chromatogram was placed onto the base layer. Then a 250 ml overlay of 2% agar containing triphenyl tetrazolium chloride (1 mg/ml) was added. The baking dish was covered with aluminum foil and was incubated at 35 C for 12 hr.

The procedure to detect flavin compounds is similar to that used for folates except that *L. casei* was used for the assay organism and Riboflavin Assay Medium was used for the base layer.

Compounds which satisfy the folate requirement of *S. faecalis* or the riboflavin requirement of *L. casei* will support the growth of the appropriate assay organism in the base layer. When the organism grows, the triphenyl tetrazolium chloride is reduced and results in a red zone on the chromatogram from which the R_f value can be determined.

Detection of low-folate mutants

S. epidermidis was grown in FAAM, diluted and spread over the surface of pour plates containing FAAM with 2% agar which had been previously inoculated with washed *S. faecalis* (10^6 organisms/ml). Colonies of *S. epidermidis* which excrete folic acid-like compounds will grow and the excreted folates will difuse into the agar. The excreted folic acid-like compounds satisfy the folate requirement of *S. faecalis* and the assay organism grows in the agar. Thus, the colonies of *S. epidermidis* which

excrete folates will have a halo of growth (about 4 mm in diameter) of *S. faecalis* below it in the agar layer. Mutants of *S. epidermidis* which excrete little or no folic acid-like compounds will have no visible halo of growth in the agar below the colony. Such mutants were picked, streaked onto FAAM, examined microscopically and then examined for the presence of catalase to differentiate between a mutant of *S. epidermidis* and any *S. faecalis* which may have been picked.

Preparation of cell-free extracts

Ballantini beads (75 to 105 microns) were added to a washed cell pellet of *S. epidermidis* until a thick, sticky paste was formed. The paste was sonicated (Raytheon, Model 101) for 2 min; the supernatant was poured off and placed in an ice bath; the paste was resuspended in 2 ml of 0.1 M tris-(hydroxymethyl)aminomethane buffer (Tris), pH 8.0; the cell suspension was sonicated for an additional 2 min after which the supernatant was removed and placed in an ice bath. This process was repeated until the supernatant lost the characteristic opalescence. The cell-free extracts were pooled and then the cell debris was removed by centrifugation at 29,000 X G for 30 min.

Protein was determined either by the colorimetric method using Biuret Reagent (22) or the ratio of ultraviolet absorption at 280 nm and 260 nm (119).

Assay for riboflavin synthetase

The activity of riboflavin synthetase in crude cell-free extracts was determined by the procedure reported by Plaut (88). The cell-free extracts were incubated with 6,7-dimethyl-8-(1'-D-ribityl)lumazine, 0.25 mM; $NaHSO_3$,

0.3 mM; potassium phosphate at pH 6.9, 16.7 mM; in a final volume of 1 ml for 60 min at 37 C. At the end of the incubation period, the quantity of riboflavin synthesized from 6,7-dimethyl-8-(1'D-ribityl)lumazine was determined by biotube assays. The following controls were used for each assay for riboflavin synthetase: to determine the amount of nonenzymatic synthesis of riboflavin from the substrate, the enzyme was omitted from the reaction mixture; to determine the amount of endogenous riboflavin in the cell-free extracts, the enzyme was denatured by boiling before adding it to the reaction mixture; and to determine the quantity of endogenous precursors, the substrate was omitted from the reaction mixture. All data have been corrected for the endogenous riboflavin activity.

Assay for pteroate synthetase

The activity of pteroate synthetase in crude cell-free extracts was determined by the procedure reported by Jones and Williams (40). The cell-free extracts were incubated with 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine, 0.05 mM; *p*-aminobenzoic acid, 1.5 mM; adenosine-5'-triphosphate, 0.4 mM; $MgCl_2$, 1 mM; ascorbic acid, 5 mM; potassium phosphate at pH 7.8, 0.1 M; in a final volume of 1 ml for 60 min at 37 C under a layer of mineral oil. At the end of the incubation period, the total quantity of pteroate compounds synthesized from the substrate was determined by biotube assays. Controls were similar to those used for the riboflavin synthetase assay to determine both nonenzymatic and endogenous folate activity. All data have been corrected for the endogenous folate activity.

Assay for GTP-cyclohydrolase

Cell-free extracts were incubated with GTP-8-^{14}C (0.3 mM, 1.5 X 10^5

DPM) and 0.1 M potassium phosphate at pH 8.0 in a final volume of 0.5 ml for 30 min at 39 C under an atmosphere of argon. The reaction was stopped with 0.1 ml of 89% formic acid and the enzyme activity was measured by the quantity of radioactive formic acid released from GTP.

Celite chromatography

Formic acid was separated from the substrate by column chromatography on Celite 535 (Johns-Manville) (102, 107). The Celite was prepared using a modification (127) of the procedure reported by Swim and Utter (111). Then the Celite (0.7 g) was acidified with 0.35 ml of 0.2 N H_2SO_4 and packed into a column (1.2 X 5.0 cm) with a glass rod. A 0.2-ml sample from the GTP-cyclohydrolase assay mixture was placed onto the column. The column was washed with 8 ml of chloroform saturated with 0.2 N H_2SO_4 and the chloroform fraction was discarded. Then the column was eluted with 1-ml fractions of 1-butanol in chloroform saturated with 0.2 N H_2SO_4 (1:4, v/v). The eluent was collected in 1-ml fractions and examined for radioactivity (see Detection of radioactivity).

Purification of GTP-cyclohydrolase

The crude cell-free extracts were incubated with ribonuclease (50 µg/ml) and deoxyribonuclease (10 µg/ml) for 20 min at 37 C. Then the extracts were heated at 60 C for an additional 20 min and centrifuged at 29,000 X G for 20 min. The supernatant was treated with 1% streptomycin sulfate (w/v) for 30 min at 4 C to precipitate the remaining nucleic acids. This mixture was centrifuged at 29,000 X G for 20 min and the precipitate was discarded. The supernatant was desalted on a column (2.5 X 90 cm) of Sephadex G-25 which had been previously equilibrated with 0.1 M Tris buffer at pH 8.0.

The initial protein peak, approximately the first 60 ml after the void volume, contained all the GTP-cyclohydrolase activity. Solid ammonium sulfate was added to the eluent to give a 60% saturated solution. The precipitate was resuspended in 15 ml of 0.1 M Tris buffer, pH 8.0, and added to a column (2.5 X 45 cm) of Sephadex G-75 and developed with 0.1 M Tris buffer, pH 8.0.

Three peaks of enzymatic activity were eluted from the Sephadex G-75 column with Tris buffer. On the basis of retention volume and added ribonuclease as a marker protein, the molecular weight of the protein in each peak was estimated to be: Peak I, greater than 75,000; Peak II, between 20,000 and 50,000; and Peak III, between 10,500 and 20,000. There was about a 200-fold purification of the GTP-cyclohydrolase enzyme in Peak I over the crude extract. Since the enzyme found in Peak I released about 5 times more free formic acid per unit weight of protein than the enzymes in either Peak II or Peak III, the partially purified enzyme from Peak I only was used for further studies. Although only a small quantity of free formic acid was released from GTP by the enzyme in either Peak II or Peak III, a second peak of radioactive formic acid was eluted from the Celite column (see Appendix Fig. 2). The second peak of radioactive formate is believed to come from a bound formyl group and is slowly cleaved and released as formate by the acid environment of the Celite column.

Heat activation of GTP-cyclohydrolase

Prior to use, 1 ml of crude cell-free extract was heated in a boiling water bath for 5 min. The denatured protein was removed by centrifugation and the protein concentration of the supernatant was determined. The

supernatant was incubated with GTP-8-^{14}C to determine the specific activity of the heat-stable GTP-cyclohydrolase. The heat treatment increased the specific activity about 10 fold, but the elution pattern of formate from Celite indicated that there was a large quantity of bound formyl as well as free formate (see Appendix Fig. 2).

Detection of radioactivity

Radioactivity, measured as disintegrations per minute (DPM), was determined by the channel ratio method in a Beckman scintillation spectrophotometer (Model DPM 100). The scintillation fluid was composed of 0.5% 2,5-diphenloxazole and 10% napthalene in 1,4-dioxane.

Meterials

Micro Inoculum Broth, Folic Acid Assay Medium and Riboflavin Assay Medium were purchased from Difco Laboratories.

Folic Acid Assay PGA Broth and Trypticase Soy Broth were purchased from Baltimore Biological Laboratories.

Napthalene and 1,4-dioxane were purchased from J. A. Baker Chemical Company.

The 2,5-diphenyloxazole was purchased from Beckman Instrument Company.

Sephadex G-25, Sephadex G-50, Sephadex G-75, Sephadex G-100, Sephadex G-200 and Blue Dextrin 2000 were purchased from Pharmacia Fine Chemicals, Inc.

Adenosine-5'-triphosphate, guanosine-5'-triphosphate, guanosine-5'-triphosphate-8-^{14}C, guanosine-5'-triphosphate-U-^{14}C, folic acid, streptomycin sulfate, tris(hydroxymethyl)aminomethane and sodium borohydride were purchased from Schwarz Biochemicals, Inc.

Ribonuclease B and deoxyribonuclease II were purchased from Worthington Biochemical Corp.

Folic acid-2-^{14}C was purchased from Amersham/Searle.

The 2-amino-4-hydroxy-6-hydroxymethyl pteridine was synthesized by L. P. Jones by the method described by Baugh and Shaw (8). Sodium borohydride was used to reduce 2-amino-4-hydroxy-6-hydroxymethylpteridine to 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine (104). The N^{10}-formylpteroic acid was synthesized by L. P. Jones using the method of Wolf, *et al.* (128).

The 2-amino-4-hydroxy-6-formylpteridine and the 2-amino-4-hydroxy-6-carboxypteridine were synthesized by F. D. Williams using the method of Waller, *et al.* (118).

The 2-amino-4-hydroxy-6-(D-*erythro*-1',2',3'-trihydroxypropyl)pteridine (D-neopterin) was synthesized by M. LeVake using the method of Rembold and Metzger (91) and purified by the chromatographic method of Rembold and Bushmann (90). The 2-amino-4-hydroxy-6-(L-*erythro*-1',2',3'-trihydroxypropyl)-pteridine (L-neopterin) was a gift from G. W. Kidder.

Leucopterin, xanthopterin, isoxanthopterin and 6,7-dimethyl-8-(1'-D-ribityl)lumazine were purchased from Fluka Chemische Fabrik.

The 2-amino-4-hydroxypteridine and 2-amino-4-hydroxy-6,7-dimethylpteridine were purchased from Regis Chemical Company.

The lumazine was purchased from Aldrich Chemical Company, Inc.

The biotin was purchased from Nutritional Biochemical Company.

RESULTS

Preliminary studies showed that *S. epidermidis* excretes into the culture medium large quantities of both folic acid-like compounds, which will support the growth of *S. faecalis*, and riboflavin-like compounds, which will support the growth of *L. casei*. The morphological and biochemical characteristics of *S. epidermidis* have remained essentially unchanged during this investigation with the exception of a change in two types of folic acid-like compounds that are excreted (see next paragraph).

Folate and riboflavin excretion by *S. epidermidis*

S. epidermidis was grown in 1.5 l of Brain Heart Infusion broth (BHI), FAAM and synthetic medium in 2.8 l Fernbach flasks with agitation on a gyrotory shaker. At itervals, a 100-ml sample was removed and centrifuged. The supernatant was examined for excreted folic acid-like compounds and excreted flavins. The cell pellet was washed and the intracellular folates and flavins were extracted. The quantity of intracellular and extracellular folates and flavins was determined by tube bioassay. The types of compounds excreted into the growth medium were separated by ascending paper chromatography in Solvent A (see Appendix Table 2 for R_f values of standard compounds) and located by bioautography. Table 1 shows the R_f values of the excreted folic acid-like compounds which are biologically active for *S. faecalis* and the excreted flavin derivatives which are biologically active for *L. casei*. Riboflavin (R_f 0.36) and traces of flavin mononucleotide (FMN) are the only flavin derivatives excreted; whereas pteroic acid (R_f 0.01), a compound thought to be pteroate pyrophosphate (R_f 0.79) and rhizopterin derivatives (R_f 0.47 and 0.61) are the typical folic acid-

Table 1. The R_f values in Solvent A for the biologically active folate and flavin derivatives excreted into the culture medium by *S. epidermidis* in the stationary phase of growth.

Growth Medium	Folic Acid-like Derivatives	Flavin Derivatives
BHI	0.60 0.01	0.36
FAAM	0.79 0.59 0.48 0.01	0.36
Synthetic	0.79 0.59 0.47 0.01	0.56 0.36

like compounds excreted. But, when *S. epidermidis* is continuously subcultured in the synthetic medium, the R_f 0.79 compound is lost with the concomitant appearance of a compound with an R_f 0.11 (believed to be a rhizopterin derivative). In the synthetic medium, the concentration of each pteroate derivative changes during growth. In the accelerated phase and early exponential phase of growth only pteroic acid is found. During exponential growth, the pteroate concentration decreases and compounds with an R_f of 0.38 and 0.47 appear in relatively high concentration. Then during the decelerated exponential phase and stationary phase of growth, the R_f 0.38 compound disappeares completely with increasing concentrations of pteroic acid and compounds with an R_f of 0.56 and 0.79. Since the biotube assay does not distinguish between these various derivatives and the standard curve is prepared with authentic folic acid, the quantity of folic acid-like compounds will be expressed as 'folate equivalents' and the

compounds will be referred to as folates. But, it should be understood that folic acid *per se* (R_f 0.35) is not found in the culture supernatant.

Fig. 1 and 2 show the intracellular and extracellular quantities of both folate and riboflavin during the growth of *S. epidermidis* in BHI and FAAM. Biotube assays were used to determine the quantity of endogenous folate and riboflavin in the BHI medium and the quantity of riboflavin in the FAAM. The extracellular values for folate and riboflavin shown in Fig. 1 and the extracellular values for riboflavin shown in Fig. 2 have been corrected for the amounts of vitamins initially present in the media. Fig. 3 shows only the folate levels observed during growth of *S. epidermidis* in the synthetic medium. The graphic presentation of the riboflavin levels was omitted because there were no samples examined for riboflavin during the mid-exponential phase of growth. The shape of the riboflavin curves are similar to the shape of the curves for cells grown in FAAM. However, in the stationary phase of growth, the intracellular concentration of flavins is 5.2 times greater than the extracellular concentration and the total flavins excreted into the synthetic medium is about one-fifth as much as the total flavins excreted into the FAAM.

The quantity of excreted folates always exceeds the intracellular quantity of folates (Fig. 1, Fig. 2, Fig. 3). In addition, the amount of excreted folates is a close approximation of the total quantity of folates synthesized, especially for cultures in the stationary phase of growth. However, the quantity of intracellular riboflavin from cultures in the stationary phase of growth approaches or exceeds the quantity excreted into the culture medium. During growth, the intracellular concentration of riboflavin remains constant, but the folate concentration does not remain

Fig. 1. The quantities of intracellular folates (▣), excreted folates (◉), intracellular riboflavin (□) and excreted riboflavin (○) synthesized by *S. epidermidis* during growth in BHI. A typical growth curve for *S. epidermidis* in BHI is shown by the solid line.

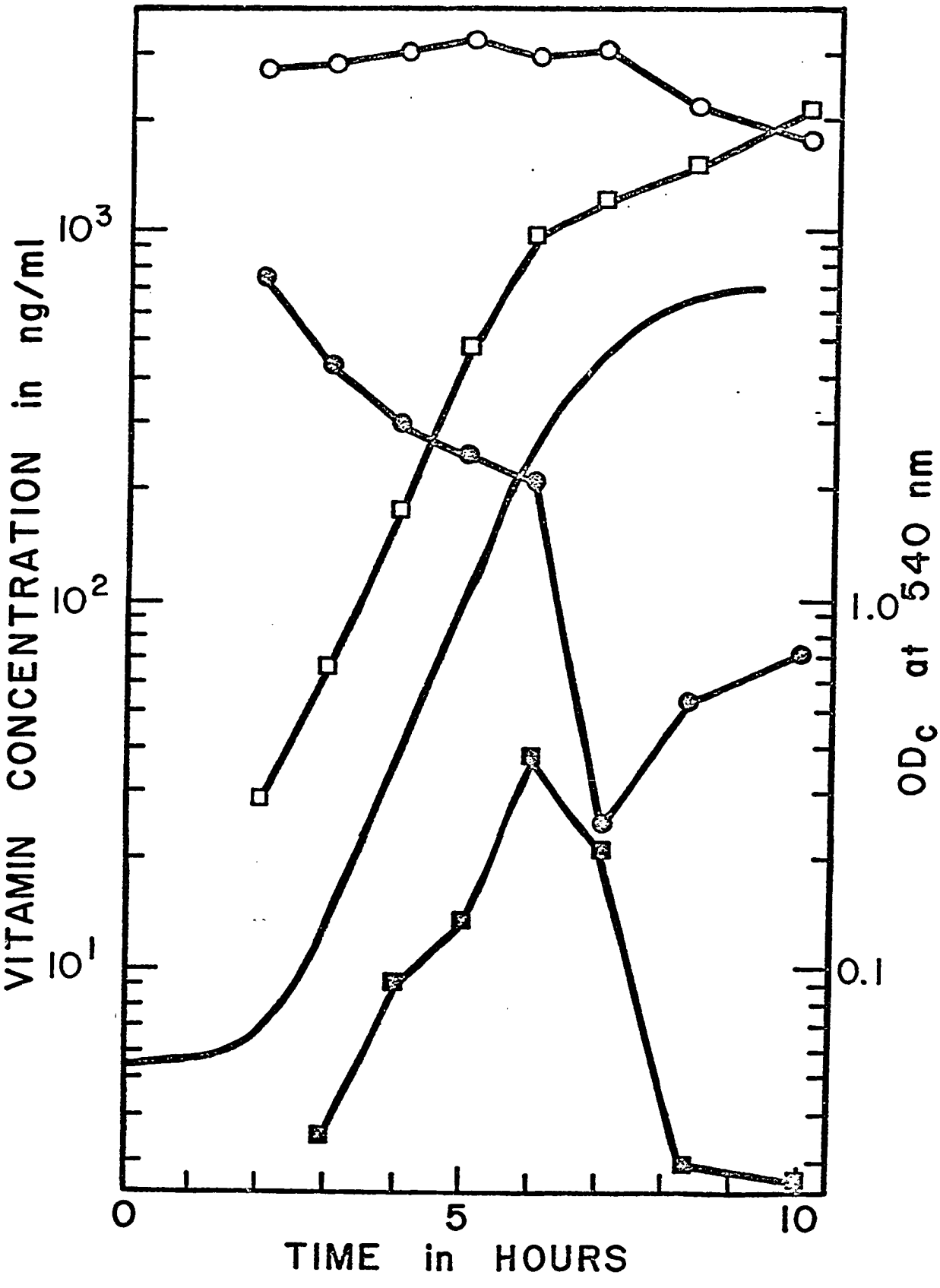

VITAMIN CONCENTRATION in ng/ml
10^3
10^2
10^1
OD$_c$ at 540 nm
1.0
0.1
0
5
10
TIME in HOURS

Fig. 2. The quantities of intracellular folates (■), excreted folates (●), intracellular riboflavin (□) and excreted riboflavin (○) synthesized by *S. epidermidis* during growth in FAAM. A typical growth curve for *S. epidermidis* in FAAM is shown by the solid line.

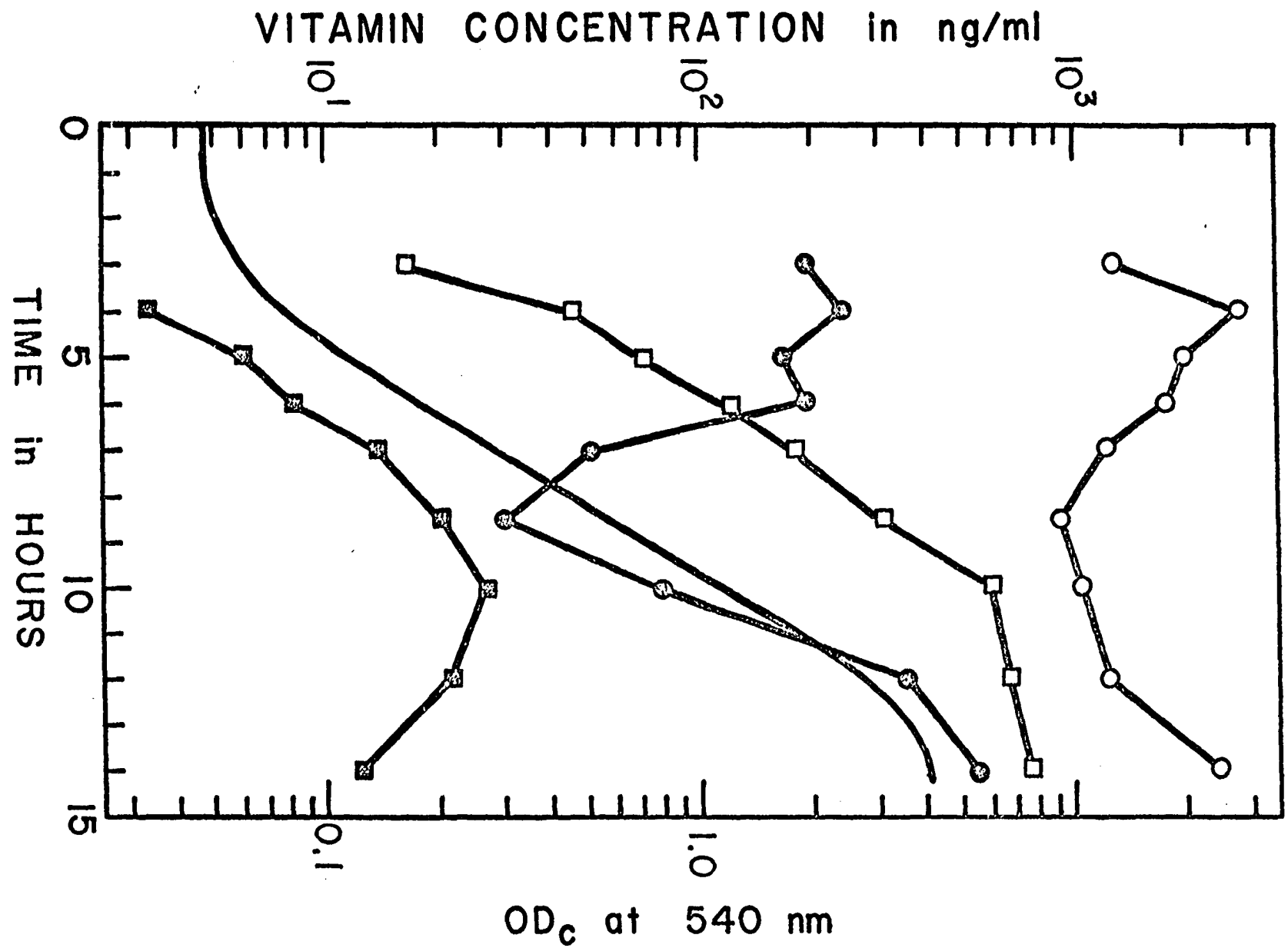
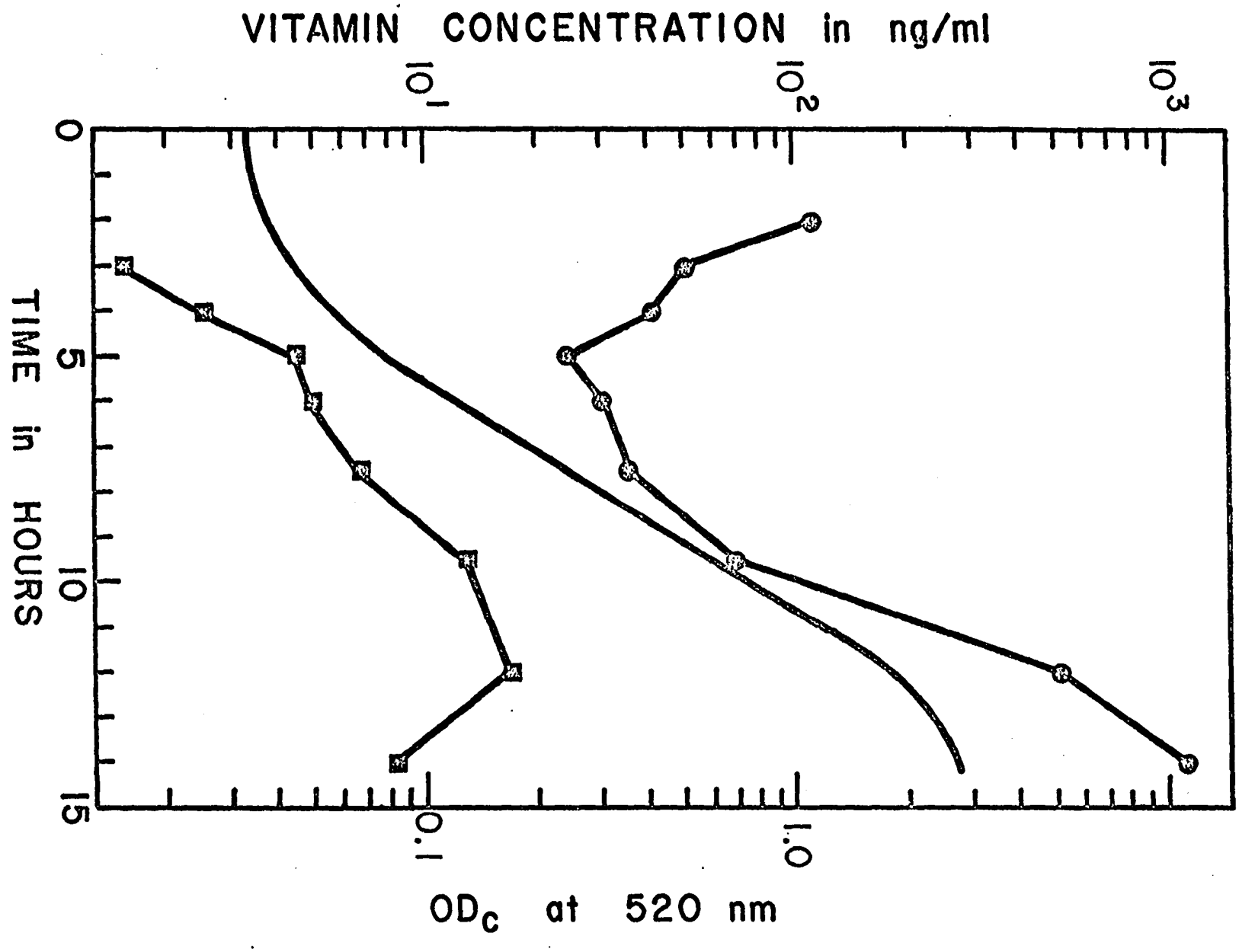

VITAMIN CONCENTRATION in ng/ml
10^{-1}
10^{2}
10^{3}
0
5
10
15
TIME in HOURS
.1
1.0
OD_c at 540 nm

Fig. 3. The quantities of intracellular folates (■) and excreted folates (●) synthesized by *S. epidermidis* during growth in the synthetic medium. A typical growth curve for *S. epidermidis* in the synthetic medium is shown by the solid line.


VITAMIN CONCENTRATION in ng/ml
10_1
10_2
10_3
0
5
10
15
TIME in HOURS
.1
1.0
OD_c at 520 nm

constant. Also, there is an apparent loss of folate activity from the culture supernatant during exponential growth which can not be explained by just uptake of folates into the cell. Either the folate compounds are taken up and are bound or they are altered to compounds such as unconjugated pteridines or pteroyl-γ-polyglutamates which are not biologically active for *S. faecalis*. Since *L. casei* will respond to the pteroyl-γ-polyglutamates as well as to folic acid and formylated folate but not to the pteroate derivatives, the spent culture medium was examined for biological activity with both *L. casei* and *S. faecalis*. Table 2 shows the absence of either pteroyl-γ-polyglutamates or folic acid in the culture medium during exponential growth and early stationary phase of growth. But, in late stationary and early death phase, compounds other than folic acid that are active for *L. casei* but not *S. faecalis* are excreted in increasing quantities apparently at the expense of the pteroate compounds.

To determine whether folic acid could enter the cell and be either bound or altered, *S. epidermidis* was grown to stationary phase in 100 ml

Table 2. The absence of extracellular folic acid and pteroyl-γ-polyglutamates in the culture medium of young cultures of *S. epidermidis* grown in FAAM.

Age of Culture in hours	Folate Equivalents in ng/ml	
	S. faecalis	*L. casei*
4	85	0
6	15	0
8	3	0
18	34	0
30	191	4
96	331	101
120	184	235

of synthetic medium supplemented with folic acid-2-^{14}C (10 μCi; 0.8 μg/ml). The culture supernatant was removed by centrifugation and examined for radioactive compounds. The cells were washed, resuspended in 2.5 ml of water and the intracellular folates were extracted. The extracted cells were washed, resuspended in 2.0 ml of water and examined for bound folates. Based upon the quantity of radioactivity recovered in each fraction, it was calculated that 77.8 μg of the originally supplemented folate remained in the culture medium while 0.448 μg could be extracted and 1.34 μg was bound. The bound radioactivity could be released with acid hydrolysis in 6.1 N HCl at 121 C for 1 hr. To determine whether the folic acid was changed, a 0.1 ml aliquot from each fraction was spotted onto Whatman No. 3MM paper and developed by ascending chromatography in Solvent A and Solvent B. The chromatograms were examined for fluorescence under UV light (254 nm), radioactivity and biological activity for *S. faecalis*. Table 3 shows the distribution of radioactivity between and within each fraction as well as the R_f values in Solvent A for the biologically active compounds for *S. faecalis*. It is clear that folic acid can enter the cell; it can be altered to pteroate derivatives; and it can be bound. No unconjugated pteridines were detected on the basis of fluorscence under UV light or on the basis of the R_f values of the radioactive compounds.

The levels of excreted folates are 20 to 100 times higher than intracellular folate levels in stationary phase cultures of *S. epidermidis*. Assuming that a large extracellular to intracellular ratio (E/I) indicates that a biochemical pathway is not subject to exact regulation, then the folate pathway in *S. epidermidis* strain W is not subject to exact regulation. If this assumption is correct, then it would follow that an organism

Table 3. The distribution of bound, intracellular and extracellular radioactive compounds derived from folic acid when *S. epidermidis* is grown in synthetic medium supplemented with folate-2-^{14}C.

Fraction	Distribution of Radioactivity Between Fractions in %	R_f values of Biological Activity Solvent A	Distribution of Radioactivity Within Fractions in %
Uninoculated Medium	100	0.00	0.1
		0.35	97.7
Spent Culture Medium	97.3	0.00	4.1
		0.43	69.4
		0.61	16.2
		0.78	6.5
Extractable Folates	0.56	0.00	18.9
		0.36	39.8
		0.60	20.1
		0.79	11.9
Bound Folates	1.67		
Released by acid hydrolysis		0.00	7.1
		0.49-0.65	88.3

which excretes little or no folate would be subject to a more exact regulation. Based upon these assumptions, I looked for either a strain or a mutant of *S. epidermidis* which would excrete little or no folic acid-like compounds. Eight strains of *S. epidermidis* were obtained from R. W. Brown, the National Animal Disease Laboratories (NADL), Ames, Iowa. The growth characteristics, the types of folic acid-like compounds excreted and the quantity of folates excreted from the NADL strains were compared to *S. epidermidis* strain W and two mutants derived from strain W (mutant NFA and mutant NFB). All the strains of *S. epidermidis* that were examined and that will grow in the synthetic medium excrete an excess of the same types of

folic acid-like compounds (See Appendix Fig. 3, Appendix Table 3, Appendix Table 4).

The 29 apparent mutants of strain W are naturally present in the culture of *S. epidermidis* at a frequency of 1 per 10^4 organisms when *S. epidermidis* strain W is continously subcultured in FAAM. The original isolates excreted less than 0.75 ng/ml of folates into the growth medium. The mutants designated NFA and NFB are characteristic of the whole group of mutants isolated. All 29 isolates revert to the wild type excretion pattern when grown in synthetic medium.

A comparison of the relative quantities of folate and riboflavin excreted by the mutant NFA and the wild type grown in FAAM (Table 4) and in synthetic medium (Table 5) suggest that the folate and riboflavin pathways are inversely related and are adaptive. First, there is an inverse relationship between the quantity of folate and riboflavin excreted, relatively low folate-relatively high riboflavin, relatively high folate-relatively low riboflavin. Second, more riboflavin is excreted under conditions of low O_2 and high CO_2 tension, whereas, folate excretion is increased by an increased O_2 tension. And finally, there is a response to one or more nutritional components in FAAM which reduces the folate synthesis and stimulates the riboflavin excretion. Since the mutants could not be distinguished from the wild type organism when grown in synthetic medium, they were not considered to be under strict regulation as originally presumed. Rather, I believe they are extreme examples of the adaptation of the folate pathway in response to some nutritional component. Therefore, information about the types of nutritional compounds which cause changes in folate and riboflavin synthesis and the extent of the

Table 4. The quantity of folate and riboflavin excreted by *S. epidermidis* strain W and mutant NFA when grown in stationary and shake flasks of FAAM.

Organism	Shake Flask			Stationary Flask		
	Growth OD	Folate ng/ml	Riboflavin ng/ml	Growth OD	Folate ng/ml	Riboflavin ng/ml
Mutant NFA	2.8	7.1	1300	2.4	0.4	1850
Strain W	3.8	158.2	1600	3.6	292.1	1175

Table 5. The quantity of folate and riboflavin excreted by *S. epidermidis* strain W and mutant NFA when grown in stationary and shake flasks of synthetic medium.

Organism	Shake Flask			Stationary Flask		
	Growth OD	Folate ng/ml	Riboflavin ng/ml	Growth OD	Folate ng/ml	Riboflavin ng/ml
Mutant NFA	2.6	490	15	0.75	220	85
Strain W	2.9	612	22	1.37	228	150

changes seemed to be required to define the extent of adaptation of these biosynthetic pathways.

Evidence for adaptive folate and flavin pathways

To determine which nutritional components of FAAM will stimulate the riboflavin excretion and reduce the folate excretion, the citrate, vitamins and purine-pyrimidine components of FAAM were added to the synthetic medium so the final concentration of each would be the same as in FAAM: sodium citrate, 10 mg/ml; thiamine hydrochloride, 1 µg/ml; pyridoxine hydrochloride,

2 µg/ml; riboflavin, 1 µg/ml; *p*-aminobenzoic acid, 0.1 µg/ml; biotin, 0.0004 µg/ml; calcium pantothenate, 0.2 µg/ml; adenine sulfate, 10 µg/ml; guanine hydrochloride, 10 µg/ml; and uracil, 10 µg/ml. *S. epidermidis* was grown in shake flasks to the stationary phase of growth in synthetic medium supplemented individually or in combination with either sodium citrate, a vitamin mixture or a purine-pyrimidine mixture. Then the culture medium was examined for the quantity of excreted folates and riboflavin (Table 6). Since the final population of *S. epidermidis* in each growth medium differs, the quantities of excreted folates and riboflavin are corrected for growth. Viable plate counts were found to be an inadequate measure of growth since

Table 6. The effects upon growth and upon the folate and riboflavin excretion of *S. epidermidis* when grown to stationary phase in synthetic medium supplemented with the components of FAAM.

Additions to the Growth Medium	Growth OD_c	Folate		Riboflavin	
		ng/ml	ng/ml/OD_c	ng/ml	ng/ml/OD_c
None	2.45	345	141	83.2	34
Vitamin Mixture	4.90	113	23	89.2	18
Citrate	0.17	14	84	47.5	276
Purine-pyrimidine	0.09	25	289	56.2	647
Vitamin Mixture + Citrate	2.72	68	25	225.5	87
Vitamin Mixture + purine-pyrimidine	5.4	124	23	460.5	82
Purine-pyrimidine + Citrate	0.10	19	195	52.5	526
Vitamin Mixture + Purine-pyrimidine + Citrate	3.4	51	15	995.0	293

S. epidermidis can grow predominately either as single organisms, in pairs, in tetrads or in clusters depending upon the phase of growth and the growth medium. Therefore, growth is measured in terms of corrected optical density (OD_c) which is proportional to mass (see Appendix Fig. 1). After corrections for growth it becomes evident that when growth is poor, the organism excretes a relatively large quantity of folate and riboflavin per organism. But, relatively small quantities per organism are excreted under conditions which allow for nearly maximal growth. In addition, there is something in the purine-pyrimidine mixture which stimulates both folate and riboflavin synthesis and something else in the vitamin mixture which decreases the synthesis of both. For folate synthesis, the vitamin effect completely negates the stimulatory effect of the purine-pyrimidine mixture. But for riboflavin synthesis, the stimulation by the purine-pyrimidine mixture overcomes the inhibition caused by the vitamin mixture.

In an attempt to find defined media which will allow *S. epidermidis* to synthesize relatively high quantities of both folate and riboflavin, relatively low quantities of both folate and riboflavin, relatively low folate-high riboflavin and relatively high folate-low riboflavin, each vitamin (Table 7) and each purine and pyrimidine (Table 8) found in FAAM was added separately and as a mixture to the synthetic medium. Xanthine, hypoxanthine, inosine, and 2,4,5-triamino-6-hydroxypyrimidine were also individually supplemented into the synthetic medium at a concentration of 10 µg/ml (Table 8). The data were corrected for growth and the total synthesis is expressed in terms relative to the quantity per corrected optical density of the unsupplemented synthetic medium. Again, it is evident that when growth is poor, the organism excretes a relatively large

Table 7. The effects upon growth and upon the folate and riboflavin excretion of *S. epidermidis* when grown to stationary phase in synthetic medium supplemented with vitamins.

Additions to the Growth Medium	Growth OD_c	Folate ng/ml/OD_c	Folate Relative Synthesis	Riboflavin ng/ml/OD_c	Riboflavin Relative Synthesis
None	2.60	149	1.00	37.4	1.00
p-aminobenzoate	0.68	262	1.75	50.3	1.34
Pyridoxine·HCl	0.73	242	1.62	149.5	4.00
Riboflavin	1.82	213	1.43	-[a]	-
Ca-Pantothenate	2.40	141	0.95	39.3	1.05
Thiamine·HCl	3.00	115	0.78	35.8	0.96
Biotin	4.20	3	0.02	3.7	0.10
Vitamin Mixture	4.40	22	0.15	20.2	0.54

[a]Not determined.

quantity of folate and riboflavin per organism and a relatively small quantity of both when growth is excellent. The additions of *p*-aminobenzoic acid, pyridoxine hydrochloride, uracil and xanthine lowered the initial pH of the synthetic medium from pH 7.4 to 7.1. I don't believe that pH alone was responsible for the decreased growth since similar results were obtained when the pH was adjusted to 7.4. Of the vitamin compounds tested, biotin strongly stimulates growth and drastically reduces both folate and riboflavin synthesis. It is also interesting that pyridoxine hydrochloride stimulates the synthesis of both folate and riboflavin. While all of the purine and pyrimidine compounds tested inhibited growth to some extent, they all stimulated the synthesis of both folate and riboflavin. While

Table 8. The effects upon growth and upon the folate and riboflavin excretion when *S. epidermidis* is grown to stationary phase in synthetic medium supplemented with purines and pyrimidines.

Additions to the Growth Medium	Growth OD_c	Folate ng/ml/OD_c	Folate Relative Synthesis	Riboflavin ng/ml/OD_c	Riboflavin Relative Synthesis
None	2.45	115	1.00	13.5	1.00
Uracil	0.10	278	2.42	177.0	13.10
Xanthine	0.19	687	5.97	156.0	11.50
2,4,5-Triamino-6-hydroxypyrimidine·SO_4	0.49	319	2.77	101.2	7.49
Inosine	0.31	284	2.47	97.5	7.22
Guanine·HCl	1.18	224	1.95	21.2	1.57
Hypoxanthine	1.72	343	2.98	17.4	1.29
Adenine·SO_4	2.10	140	1.22	17.1	1.26
Purine-pyrimidine Mixture	1.82	2.14	1.86	128.1	9.49

uracil, xanthine, inosine and 2,4,5-triamino-6-hydroxypyrimidine have a strong stimulatory effect upon the folate and riboflavin excretion, it is suprising that guanine has so little effect upon the excretion of either vitamin. Table 9 lists the defined growth media and the desired characteristics related to folate and riboflavin synthesis which will be used to determine the rates of synthesis and specific activities of selected enzymes.

S. epidermidis was grown in 2.8 l Fernbach flasks containing 1.5 l of synthetic medium, synthetic medium supplemented with 4 ng/ml of biotin and

Table 9. A summary of the effects of the growth medium upon the relative growth and the relative quantity of folates and riboflavin synthesized when *S. epidermidis* is grown to the stationary phase.

Growth Medium	Relative Growth	Relative Folate Synthesis	Relative Riboflavin Synthesis
Synthetic + Xanthine	Very Poor	High	High
Synthetic	Good	High	Low
FAAM	Good	Low	High
Synthetic + Biotin	Excellent	Low	Low

FAAM. The synthetic medium supplemented with xanthine was omitted because it would not support adequate growth of *S. epidermidis* for this experiment. At intervals during growth, samples were removed, growth was determined by corrected optical density, dry weight and viable plate counts. Then the culture supernatant was examined for excreted folates and riboflavin and the cell pellet was extracted to remove the intracellular folates and riboflavin. The total quantity of folate and riboflavin (extracellular plus intracellular) is expressed in moles (nmoles/ml) and is divided by the mass (mg dry weight/ml) so that the total synthesis is expressed as nmoles/mg dry weight and is plotted against mass doubling (Fig. 4 and Fig. 5). The generation time of *S. epidermidis* is: 28 min in the biotin supplemented medium; 37 min in the FAAM; and 52 min in the synthetic medium. The data are distorted if the quantity per cell is plotted against time since the organisms grown in the biotin supplemented medium are in the stationary phase of growth at the same time that the organisms grown in the synthetic

Fig. 4. The total folate synthesis per cell related to growth of *S. epidermidis* in the synthetic medium (△), the biotin supplemented medium (○) and the FAAM (□).

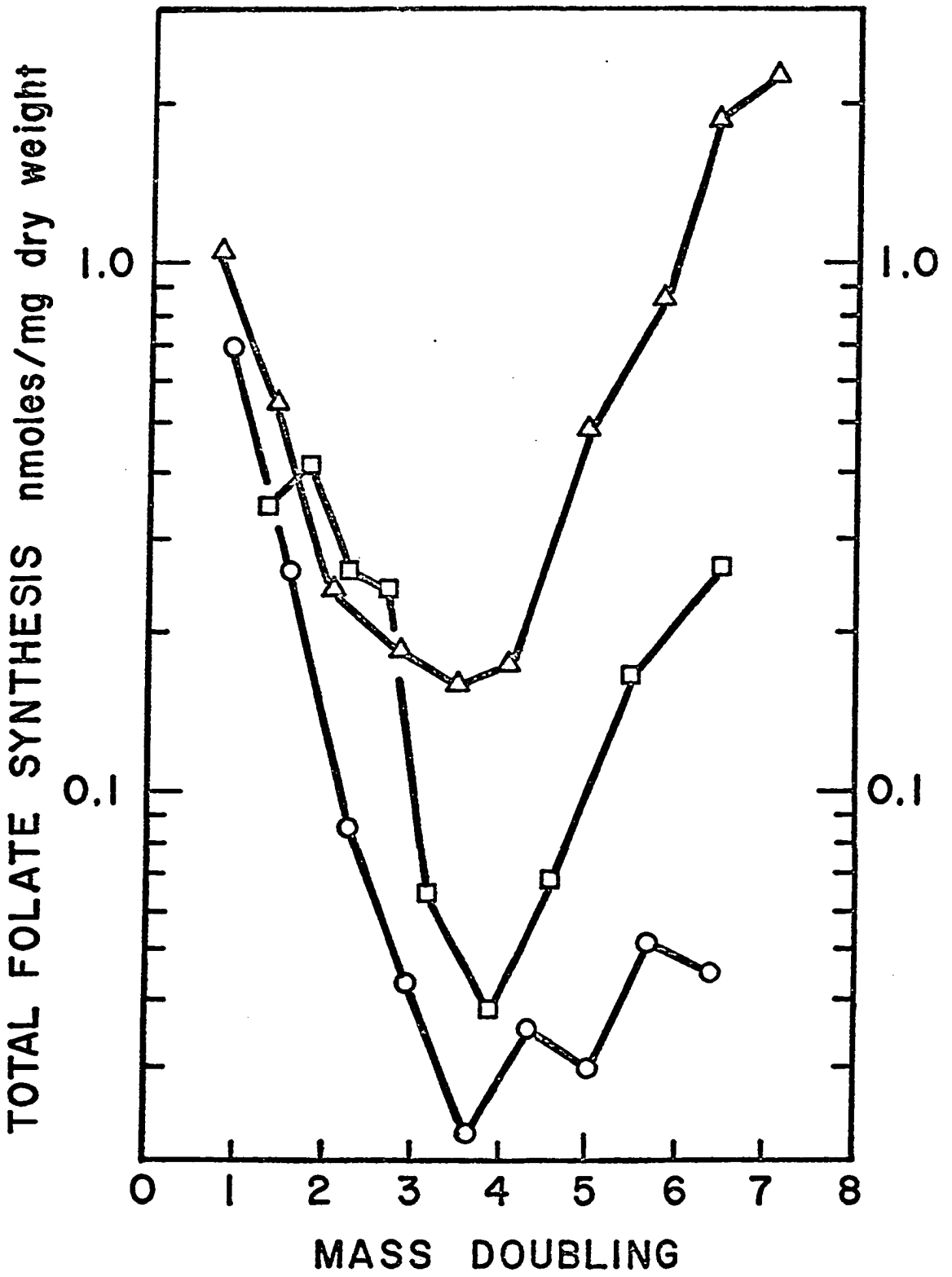
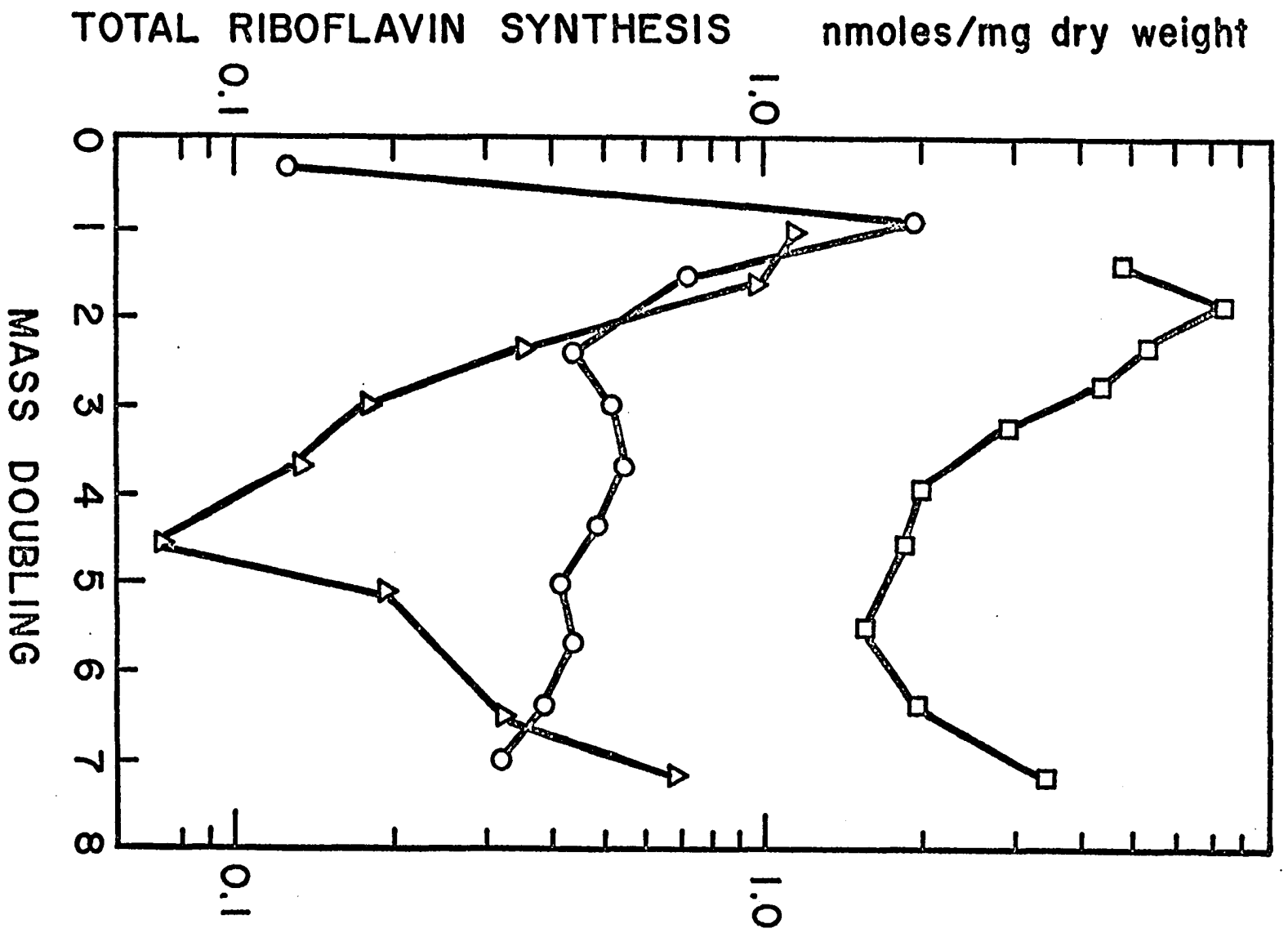

TOTAL FOLATE SYNTHESIS nmoles/mg dry weight
1.0
0.1
1.0
0.1
0 1 2 3 4 5 6 7 8
MASS DOUBLING

Fig. 5. The total riboflavin synthesis per cell related to growth of *S. epidermidis* in the synthetic medium (△), the biotin supplemented medium (○) and the FAAM (□).


TOTAL RIBOFLAVIN SYNTHESIS nmoles/mg dry weight
0.1
1.0
MASS DOUBLING
0
1
2
3
4
5
6
7
8
0.1
1.0

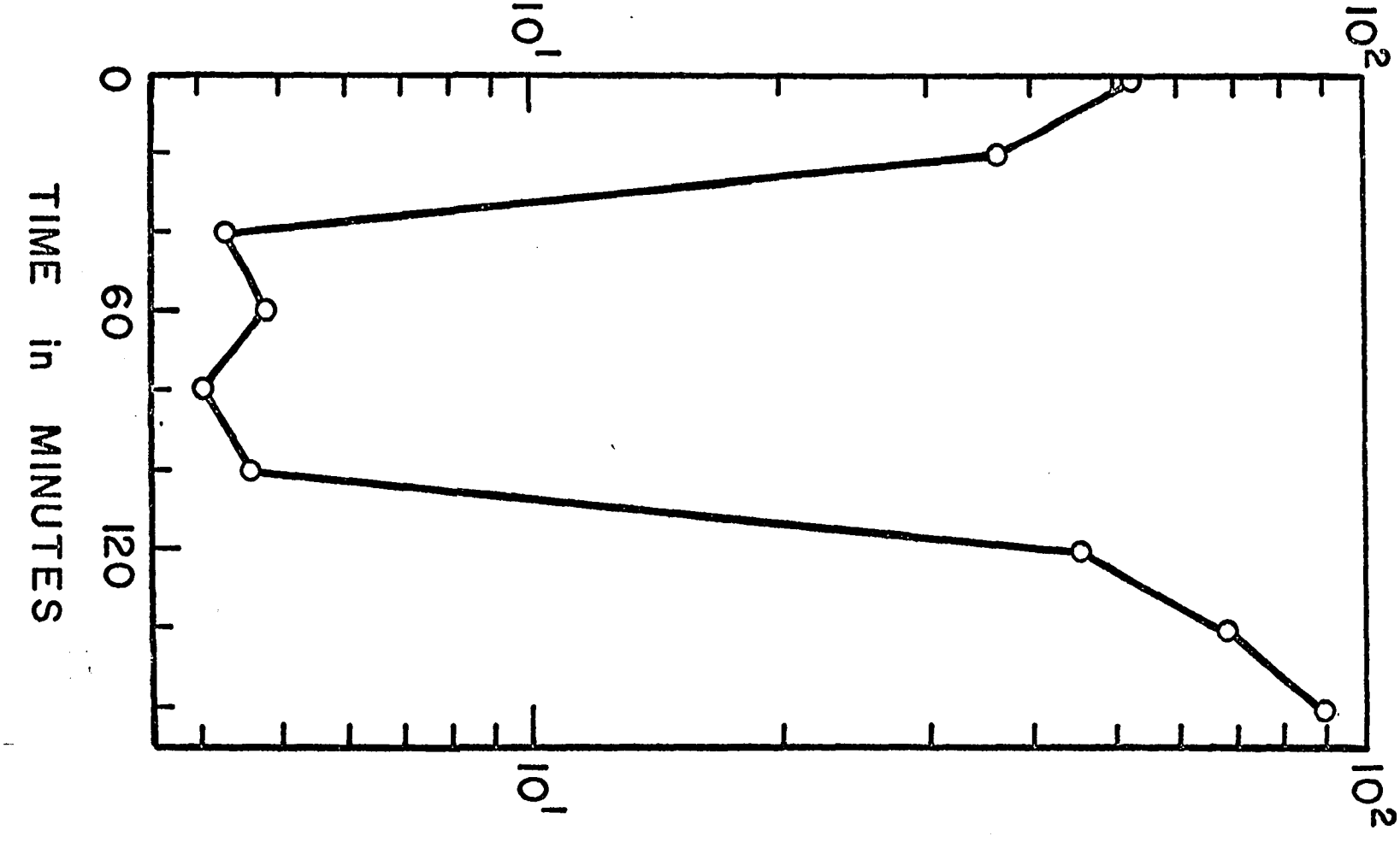
medium are in the exponential phase of growth. As pointed out previously, viable plate counts were an inadequate measure of growth. Therefore, the data are plotted as the quantity per mass against the doubling of mass in order to approximate a quantity per cell per generation. If the enzymes of either the folate biosynthetic pathway or the riboflavin biosynthetic pathway are constitutive and have a constant rate of synthesis per cell, then the quantity/mass plotted against mass doubling should be a straight line with zero slope. But it is clear from Fig. 4 and Fig. 5 that the rate of synthesis changes for both folate and riboflavin with the possible exception of riboflavin synthesis during exponential and stationary phases of growth in the biotin supplemented medium. The data indicate that both folate and riboflavin are synthesized in excess during the lag phase, then synthesis stops or is severly reduced with concomitant dilution of the specific vitamins per cell during early exponential phase and finally synthesis is again initiated about late exponential phase of growth and continues through the early stationary phase.

To further substantiate the time of vitamin synthesis in relation to growth, the specific activity of GTP-cyclohydrolase, the first enzyme complex in the folate pteridine biosynthesis, was examined during exponenial growth (Fig. 6). At 20 min intervals, starting at early exponential growth, samples of *S. epidermidis* were taken from the synthetic medium and the cells were disrupted. The cell-free extracts were heated in a boiling water bath to activate GTP-cyclohydrolase (see Appendix Fig. 2). When the specific activity, expressed in nmoles of formate released from GTP/mg protein/ml/hr, of GTP-cyclohydrolase was measured, it was found to decrease during early exponential growth followed by an increase during late

Fig. 6. The specific activity of heat treated GTP-cyclohydrolase prepared from crude cell-free extracts taken during the exponential phase of growth in the synthetic medium.

SPECIFIC ACTIVITY of heat treated GTP-CYCLOHYDRLASE

nmoles formate/mg protein/ml/hr



exponential growth. This correlates with the information on the rate of total synthesis and reinforces the interpertation that folate synthesis is reduced and then later initiated in excess during this period of growth.

Evidence for a branched pteridine pathway

By taking advantage of the effect of the growth media upon folate and riboflavin biosynthesis, the specific activity of enzymes from the pteridine, folate, and riboflavin pathways were examined. The following enzymes were selected because there are adequate assay systems for each and because each enzyme is known to participate in the synthesis of compounds in the pathways in question: GTP-cyclohydrolase, the first enzyme complex in the pteridine pathway, cleaves C-8 of GTP and releases it as formate; pteroate synthetase, which is two enzymes, adds a pyrophosphate moiety to 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine and then adds *p*-aminobenzoic acid to form pteroic pyrophosphate in *S. epidermidis*; and riboflavin synthetase, the terminal enzyme in riboflavin synthesis, makes one molecule of riboflavin from two molecules of 6,7-dimethyl-8-(1'-D-ribityl)lumazine. *S. epidermidis* was grown in synthetic medium, biotin supplemented medium and FAAM. The cells were collected from the late exponential phase of growth and disrupted. The crude cell-free extracts were used for the assay because one of the enzymes, pteroate synthetase, loses its activity if it is dialyzed, heated or frozen. Table 10 shows the specific activities for the enzymes representative of the pteridine, folate and riboflavin pathways and the total quantity of folate and riboflavin synthesized by the organisms grown in each medium at the time the samples were taken. There is a good correlation between the specific activity of the enzymes and the total

Table 10. The specific activity of enzymes representing the pteridine, folate and riboflavin pathways in crude cell-free extracts prepared from late exponential phase cultures of *S. epidermidis*.

Growth Medium	Specific Activity nmoles/mg protein/ml/hr			Total Synthesis nmoles/mg dry wt	
	GTP-cyclohydrolase	Pteroate Synthetase	Riboflavin Synthetase	Folate	Riboflavin
Synthetic	4.100	0.173	0.217	0.92	0.42
Synthetic + Biotin	0.260	0.006	0.114	0.03	0.19
FAAM	0.887	0.007	0.460	0.06	2.13

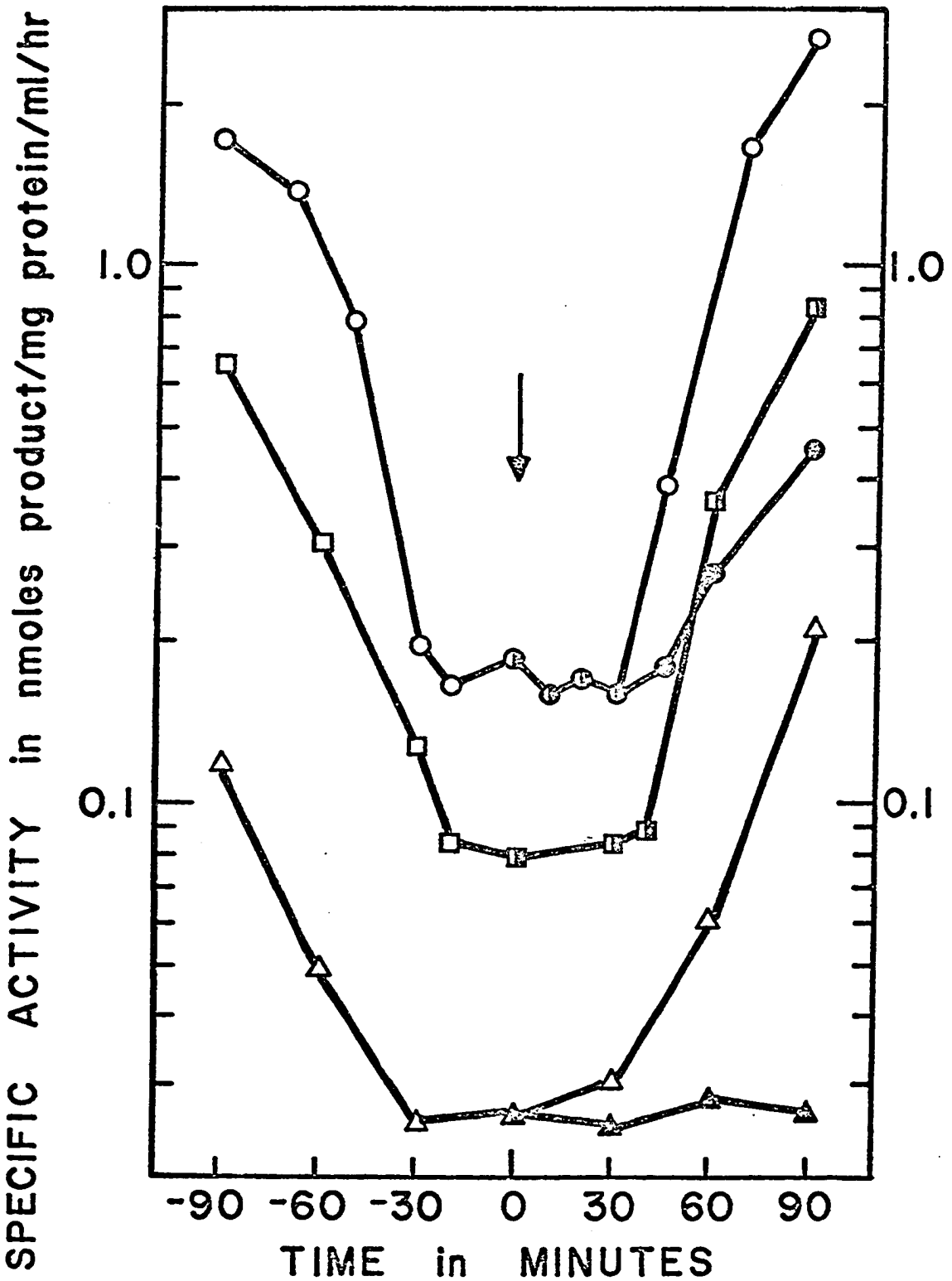
synthesis of the appropriate vitamin compound in a given growth medium. The unique feature is that GTP-cyclohydrolase activity remains relatively high even when the pteroate synthetase activity is very low (in the biotin supplemented medium) and the GTP-cyclohydrolase activity increases disproportionately with a negligible increase in the pteroate synthetase activity (in the FAAM). This suggests that a product of the GTP-cyclohydrolase enzyme may be required for something other than folate synthesis, possibly riboflavin synthesis. The variance of the specific activity of the enzymes also suggest that they must be subject to some type of regulation.

If an organism is supplied with all the products of folate metabolism (inosinic acid, thymine, methionine, serine-glycine, *etc.*) then there should be little requirement for the biosynthesis of folic acid and the folate synthesis should be at a minimum. Conversely, if the organism was grown in a medium void of the end products of folate metabolism, then there should be a greater demand for folic acid and the folate synthesis should

be at a maximum. Thus, it should be possible to shift the folate synthesis from near the maximum to the minimum rate of synthesis by the addition of one or all the products of folate metabolism. To test this hypothesis, the organism was grown in the pyruvate medium (described in EXPERIMENTAL PROCEDURE). When the cultures were in exponential growth, samples were removed at 10 min intervals, disrupted and the specific activity of GTP-cyclohydrolase, pteroate synthetase and riboflavin synthetase were measured. After 90 min of growth, the culture was equally divided into two flasks. To one of the flasks, some of the end products of folate metabolism (thymine, 50 µg/ml; thymidine, 50 µg/ml; serine, 150 µg/ml; glycine, 250 µg/ml; and methionine, 15 µg/ml) were added. The other flask served as a control. For the next 90 min, samples were removed at 10 min intervals and the specific activity of the three enzymes were measured. The experimental design was based upon the method used by Kovach *et al.* (51) to demonstrate either concomitant or sequential repression of histidine biosynthesis. In the control flask without the added inhibitors, it was found that the specific activity of all three enzymes rapidly drops, levels off and rapidly increases during exponential growth similiar to that already shown for GTP-cyclohydrolase (see Fig. 6). The only effect of the added inhibitors (Fig. 7) was to prevent an increase of the pteroate synthetase activity during the late exponential phase of growth and to slightly reduce the rate of increase of the GTP-cyclohydrolase activity. The riboflavin synthetase activity increased in late exponential phase of growth in the culture with the inhibitors at the same rate as the control flask.

In an attempt to find out if known intermediates of folate or riboflavin synthesis would decrease the specfic activity of GTP-cyclohydrolase,

Fig. 7. The effect of potential inhibitors (thymine, thymidine, glycine, serine and methionine) on the specific activity of GTP-cyclohydrolase (○), pteroate synthetase (△) and riboflavin synthetase (□). The inhibitors were added at time 0, indicated by the arrow. The closed symbols represent the specific activity of the control without inhibitors.


SPECIFIC ACTIVITY in nmoles product/mg protein/ml/hr
1.0
0.1
1.0
0.1
-90 -60 -30 0 30 60 90
TIME in MINUTES

dimethyl-8-ribityllumazine, 1 µg/ml, and hydroxymethylpteridine, 1 µg/ml, were added to the synthetic medium. The cells were collected in the late exponential phase of growth and disrupted. The crude cell-free extracts were heat treated to activate GTP-cyclohydrolase and then the specific activity of the enzyme was determined. In the second part of the experiment, the cells from the supplemented medium were washed and inoculated into the unsupplemented medium, grown to the late exponential phase and the specific activity of GTP-cyclohydrolase was again determined (Table 11). Cells grown in the biotin supplemented medium were included as a control. The specific activity of GTP-cyclohydrolase was decreased by the addition of either dimethyl-8-ribityllumazine or hydroxymethylpteridine to the growth medium. The decrease in specific activity could be partially

Table 11. The specific activity of GTP-cyclohydrolase after growth in the presence of and the subsequent removal of either 6,7-dimethyl-8-ribityllumazine (DMRL) or 2-amino-4-hydroxy-6-hydroxymethylpteridine (HMPt).

Additions to the Growth Medium	Specific Activity nmoles of formate released/mg protein/ml/hr			
	Inoculated from Synthetic into Supplemented Medium		Inoculated from Supplemented Medium into Synthetic Medium	
	Crude	Heat Treated	Crude	Heat Treated
None (control)	5.520	60.70	5.35	58.7
DMRL	0.770	0.669	4.25	24.3
HMPt	0.917	0.764	4.83	26.2
DMRL + HMPt	0.548	0.422	4.07	20.8
Biotin (control)	0.274	0.131	0.865	7.23

reversed by the removal of the affectors. The decrease in the specific activity in response to the affectors could be caused either by a reduced number of enzyme molecules per cell or by a reduced activity of the enzyme. It has been observed that heat treatment of crude cell-free extracts consistently increases the specific activity of GTP-cyclohydrolase. It is unclear whether the heat treatment releases a tightly bound inhibitor, disassembles the protein complex of GTP-cyclohydrolase into sub-units or simply appears to increase due to the precipitation of heat labile protein. Regardless of how the heat treatment functions, the important feature is that the heat treatment of GTP-cyclohydrolase in the presence of affectors such as dimethyl-8-ribityllumazine, hydroxymethylpteridine or biotin actually decreases the specific activity. When the affectors are removed or diluted, the activation of GTP-cyclohydrolase by heat is partially restored.

Attempts to demonstrate feedback inhibition of GTP-cyclohydrolase were inconclusive. There was a slight decrease (about 20% to 30%) in the specific activity of the partially purified enzyme from the first peak of the Sephadex G-75 column when neopterin, hydroxymethylpteridine, hydroxymethyldihydropteridine, pteroic acid, rhizopterin, folic acid, dihydrofolic acid, dimethyl-8-ribityllumazine, riboflavin or FMN were added individually to the reaction mixture. Similar results were obtained when ATP, inosine, thymine, thymidine, serine or methionine were added. A more pronounced decrease in the specific activity (about 80%) was observed when biotin was added to the reaction mixture. It is not known whether biotin inhibits the reaction or simply binds the one carbon unit released from GTP thus giving a deceptive appearance that the reaction was inhibited. Pyridoxal-

6-phosphate, which stimulates both folate and riboflavin synthesis in cultures, had no effect upon the release of formate from GTP in the reaction mixture. It was believed that pyridoxal-6-phosphate may participate in the purine ring-opening reaction by forming a Shiff's base. Since only one concentration of substrate and one concentration of affector was used in all of these experiments, nothing conclusive can be said about feedback inhibition of GTP-cyclohydrolase. If GTP-cyclohydrolase is subject to negative feedback inhibition, then the most probable inhibitor would be the compound just prior to the hypothetical branch point in the pteridine pathway.

The 4,5-diaminopyrimidine type compounds are presumed to be the intermediates arising from GTP for both riboflavin synthesis (5) and folate synthesis (129). The isolation of such a hypothetical intermediate followed by the enzymatic synthesis of both folates and riboflavin from it would provide the direct evidence for a branched pteridine pathway. Furthermore, the regulatory role of such a compound could then be thoroughly examined. Because of the reported instability of these types of compounds and the lack of an adequate method to follow their formation, the presence of such compounds could only be implied.

The supernatant from five 0.5-ml reactions of GTP-8-^{14}C with the partially purified GTP-cyclohydrolase were pooled. The substrate and intermediates were adsorbed onto activated charcoal and the radioactive formate was removed by filtration. The substrate and intermediates were eluted from the charcoal with a solution of 95% ethanol:concentrated NH_4OH (3:1, v/v) and dried in a vacuum oven at 60 C. The residue was resuspended in 2.5 ml of deionized water and added to a column (0.7 X 20 cm) of DEAE

cellulose. The column was washed with 50 ml of water and then eluted in a stepwise fashion with 50-ml portions of 0.05 M, 0.1 M, 0.2 M and 0.5 M ammonium acetate. The eluent was collected in 5-ml fractions and each fraction was examined for radioactivity and UV absorption at 230, 240, 250, 260 and 280 nm. If there was any UV absorption at any of the fixed wavelengths, then an absorption spectrum was determined for that sample. Four radioactive compounds were eluted from the DEAE cellulose column with the following molarities of ammonium acetate: Compound I, 0.05 M; Compound II, 0.2 M; Compound III, 0.2 M; and Compound IV, 0.5 M. Table 12 shows some characteristics of each compound. Compound IV was identified as GTP on the basis of its UV absorption spectrum, its R_f in Solvent A and Solvent B, and

Table 12. The characteristics of the compounds eluted from DEAE cellulose which originated from GTP-8-^{14}C incubated with partially puridied GTP-cyclohydrolase.

Compound	Molarity of Eluant	Radioactivity CPM	R_f value of Radioactive Compounds		UV Absorption in 0.1 N NaOH	
			Solvent A	Solvent B	Ab_{max}	Ab_{min}
Formate[a]	-[b]	21,200	-	-	-	-
Compound I	0.05	62,600	0.36	0.14	265	235
Compound II	0.2	7,800	-	0.46	258	232
Compound III	0.2	1,300	-	0.09	245	230
Compound IV	0.5	675,800	0.90	0.03	258 265	230
GTP Standard	0.5	-	0.93	0.01	258 265	230

[a]Determined by Celite chromatography.

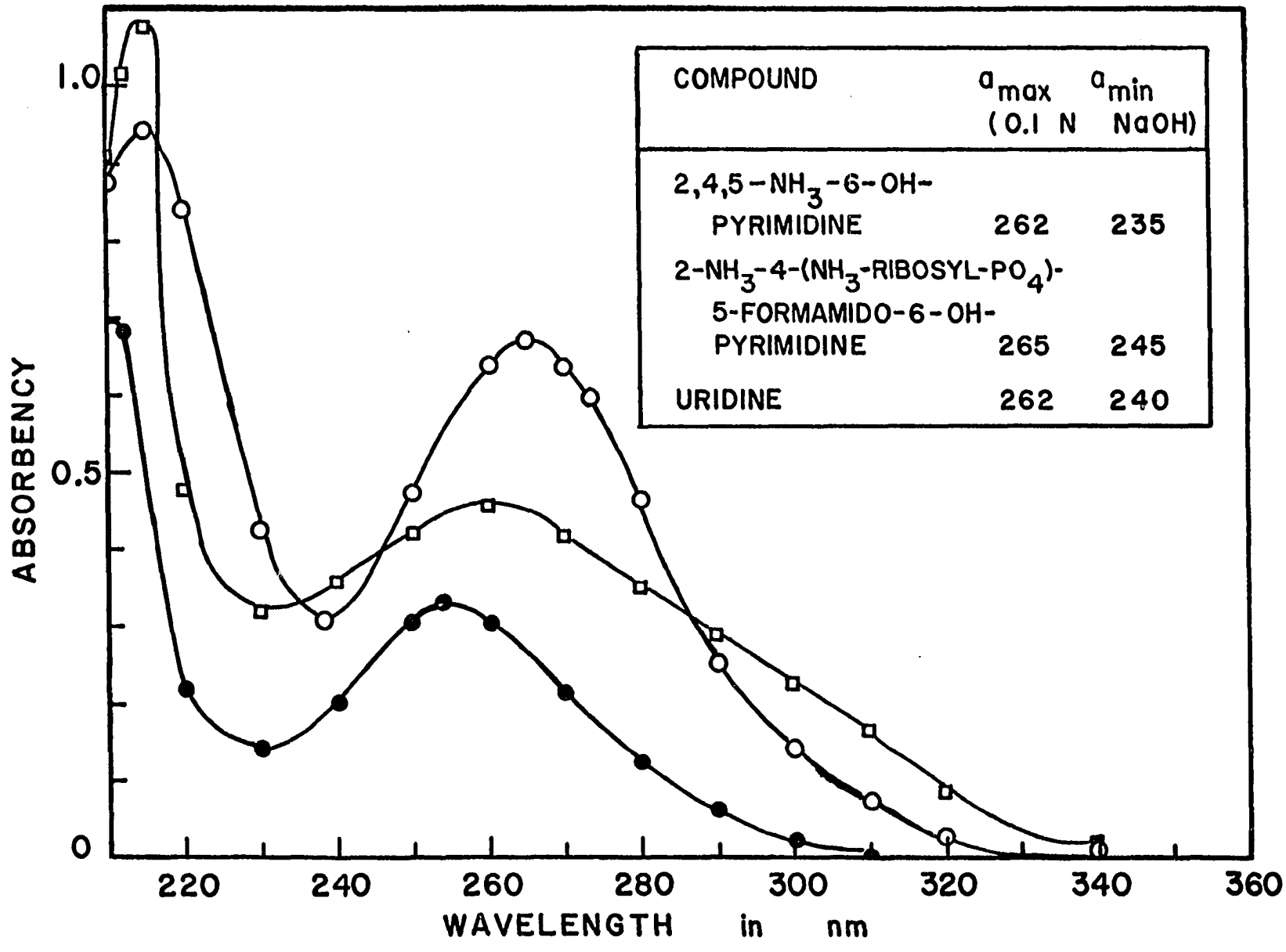
[b]Not determined.

the similarity of its elution pattern from DEAE cellulose compared with authentic GTP. Compound I and Compound II have UV absorption spectra that are characteristic of pyrimidine compounds in general and Compound I has a UV spectrum (Fig. 8) which is very similiar to the reported UV spectrum for a formamido pyrimidine compound synthesized non-enzymatically from GTP (107). The radioactive carbon atom on Compound I (originating from C-8 of GTP) is labile in 2 N H_2SO_4 and is released in the form of formic acid.

Either 2,4,5-triamino-6-hydroxypyrimidine or 4,5-diamino-2,6-dihydroxypyrimidine are known to react with glyoxal to form either 2-amino-4-hydroxypteridine (pterin) or 2,6-dihydroxypteridine (lumazine) respectively (57). And 4(1'-D-ribitylamino)-5-amino-2,6-dihydroxypyrimidine will react with diacetyl to form 6,7-dimethyl-8-(1'-D'ribitylamino)lumazine (61, 88, 89, 117, 126). In order to determine the nature of Compound I, it was reacted with diacetyl. The product was non-radioactive, had a yellow-green fluorescence under UV light (254 nm) and had R_f values of 0.18 and 0.55 in Solvent B and Solvent C respectively. The condensation product stimulates the growth of a riboflavin-deficient mutant of *Bacillus subtilis*. The riboflavin mutant of *B. subtilis* was isolated and characterized by H. R. Turner. Dimethyl-8-ribityllumazine was shown to replace the riboflavin requirement. The condensation product of Compound I appears to be 6,7-dimethyl-8-ribityllumazine. This suggests that Compound I is probably 4-ribitylamino-5-formamido-2,6-dihydroxypyrimidine or a phosphate ester of such a compound and that C-8 of GTP is retained on N-5 of the pyrimidine intermediate until after both an oxidative deamination at C-2 of the pyrimidine and a rearrangement of the ribose moiety to a ribityl moiety.

Compounds II and III could not be characterized except to note their

Fig. 8 The UV absorption spectra of Compound I from DEAE cellulose in 0.1 N NaOH (○), 0.1 M potassium phosphate buffer, pH7.0 (□) and 0.1 N HCl (●). The inset lists the reported maximum and minimum for some pyrimidine compounds.


COMPOUND
a_{max} (0.1 N NaOH)
a_{min} NaOH)
2,4,5-NH_3-6-OH-PYRIMIDINE 262 235
2-NH_3-4-(NH_3-RIBOSYL-PO_4)-5-FORMAMIDO-6-OH-PYRIMIDINE 265 245
URIDINE 262 240
ABSORBENCY
1.0
0.5
0
220
240
260
280
300
320
340
360
WAVELENGTH in nm

UV absorption maximum and minimum upon the initial separation on the DEAE cellulose column. There was a very small quantity of each and the radioactive carbon of both compounds was acid labile. Within 24 hr after the separation of these compounds, the UV spectrum of each became indistinct with no sharp absorption maximum or minimum.

Since Compound II and Compound III from the DEAE cellulose column were unstable and found in such small quantities, GTP-U-^{14}C was used as a substrate so that the intermediates would have a radioactive label. After the enzymatic reaction was stopped, the mixture was reacted with either glyoxal or diacetyl in order to make stable pteridine derivatives from any 4,5-diaminopyrimidines that were enzymatically formed. The mixture from the glyoxal condensation was added to a DEAE cellulose column. The column was washed with 50 ml of water and developed stepwise with 50 ml volumes of formic acid in increasing molarity (0.001 M, 0.005 M, 0.01 M, 0.05 M, 0.1 M, and 1.0 M). A radioactive substance was eluted from the column in the first 20 ml of the 0.001 M formic acid. The elution pattern from the column was identical to authentic pterin and the UV absorption spectrum was identical to authentic pterin. However, when the radioactive sample was chromatographed on Whatman No. 3MM paper in Solvent B, two compounds were found with R_f values of 0.35 and 0.46. Both compounds were radioactive, both had an ice blue fluorescence under UV light (254 nm), and neither compound would stimulate the growth of the riboflavinless mutant of *B. subtilis*. These compounds are assumed to be either a riboside or ribotide derivative of a pterin and a lumazine, but there are no authentic standards for comparison.

When the enzymatic products from GTP-U-^{14}C are reacted with diacetyl

and chromatographed, there are two major radioactive fluorescent compounds. One has an ice blue fluorescence under UV light, an R_f of 0.58 to 0.68 in Solvent C and will not stimulate the growth of the riboflavinless mutant of *B. subtilis*. This compound is believed to be either a riboside or ribotide derivative of 6,7-dimethylpterin, but there are no authentic standards for comparison. The other compound has yellow-green fluorescence under UV light, an R_f of 0.51-0.57 in Solvent C and strongly stimulates the growth of the riboflavin mutant of *B. subtilis*. This compound is either 6,7-dimethyl-8-ribityllumazine or a phosphate ester of such a compound. Table 13 summarizes the R_f values and the biological activity for *B. subtilis* of the compounds formed from either a glyoxal or a diacetyl condensation. The radioactive pteridine-like compounds formed by a condensation with either glyoxal or diacetyl suggests that the 4,5-diaminopyrimidine type compounds are intermediates in pteridine biosynthesis and that they originate from GTP. These pyrimidine intermediates are probably 4-(1'-D-ribitylamino)-5-formamido-2,6-dihydroxypyrimidine and 2-amino-4-(1'-D-ribosylamino)-5-formamido-6-hydroxypyrimidine or a phosphate ester of such compounds. These two intermediates would be expected if GTP was the precursor of both folates and riboflavin and if the pteridine pathway was branched or if there were isoenzymes of GTP-cyclohydrolase.

Table 13. The R_f values and the biological activity for a riboflavinless mutant of *B. subtilis* for pteridine-like compounds formed from a glyoxal and diacetyl condensation of 4,5-diaminopyrimidines.

Condensation Reaction	R_f Values Solvent B	Solvent C	Color of UV Fluorescence	Stimulation of *B. subtilis*
2,4,5-triamino-6-hydroxypyrimidine + glyoxal	0.39	0.47	Ice Blue	Negative
Pterin	0.39	0.48	Ice Blue	Negative
2,4,5-triamino-6-hydroxypyrimidine + diacetyl	0.46	0.62	Ice Blue	Negative
6,7-dimethylpteridine	0.46	0.62	Ice Blue	Negative
4,5-diaminouracil + glyoxal	0.40	0.49	Ice Blue	Negative
Lumazine	0.41	0.50	Ice Blue	Negative
4-(1'-D-ribitylamino)-5-amino-2,6-dihydroxy-pyrimidine + diacetyl	0.19	0.55	Yellow-green	Positive
6,7-dimethyl-8-ribityl-lumazine	0.19	0.55	Yellow-green	Positive
Compound I + diacetyl	0.18	0.55	Yellow-green	Positive
Enzymatic Products from GTP-U-^{14}C + glyoxal	0.35	-[a]	Ice Blue	Negative
	0.46	-	Ice Blue	Negative
Enzymatic Products from GTP-U-^{14}C +diacetyl	0.14-0.20	0.51-0.57	Yellow-green	Positive
	0.42-0.53	0.58-0.68	Ice Blue	Negative

[a]Not Determined.

DISCUSSION

This investigation has revealed two significant features about folate and riboflavin biosynthesis in *Staphylococcus epidermidis*. First, there is a change or an adjustment of both folate and riboflavin biosynthesis in response to the physiological changes that occur during growth in a given culture medium and in different culture media. Second, under all experimental conditions that were used, *S. epidermidis* always synthesizes and excretes more folate or riboflavin than is required by the organism for growth. The first feature, the adjustment of biosynthesis in response to physiological changes, is important in making a distinction between constitutivity and an elaborate control mechanism where either the quantity or the activity of the biosynthetic enzymes change in response to the physiological environment. The extent of adjustment and the rate of adjustment of either folate or riboflavin biosynthesis helps to differentiate between an induction-repression mechanism and a feedback inhibition mechanism. The second feature, overproduction, is a measure of the precision of adjustment. In describing a regulatory mechanism for folate or riboflavin biosynthesis in *S. epidermidis*, both adjustment to physiological changes and overproduction must be considered.

Adjustment of folate and riboflavin biosynthesis to physiological changes

Pardee and Beckwith (82) imply that the enzymes responsible for the synthesis of minor metabolites are constitutive and have a constant and excessive rate of synthesis per organism. If the enzymes for the minor pathways are constitutive, then the following characteristics would be expected: the total synthesis of either folates or riboflavin (intracellular

plus excreted) would increase at a constant rate during growth and the concentration of vitamins would parallel growth; the total synthesis per cell per generation would be constant; the specific activity of an enzyme in the given pathways would be constant; and the vitamins would always be overproduced resulting in excretion of both folates and riboflavin. But, if the enzymes for the minor pathways have an elaborate control mechanism, then the total quantity of folates and riboflavin should vary significantly in response to physiological changes; the total synthesis per cell per generation should not be constant; the specific activity of GTP-cyclohydrolase, pteroate synthetase and riboflavin synthetase should vary during growth in different culture media; and the end product should not be overproduced.

The rate of enzyme formation can vary by a factor of 1000 or more (19, 38, 99) for some enzymes which are subject to induction-repression. But the rate of enzyme formation has been reported to vary from 10- to 30-fold for several enzymes of the uracil (9), tryptophan (130) and histidine (2) pathways. There is some uncertainty in determining whether an enzyme is truly constitutive or subject to induction-repression when the specific activity of an enzyme remains relatively constant under a variety of growth conditions (82). When differentiating between constitutivity and induction-repression in systems where the rate of enzyme synthesis does not change dramatically, it is necessary to consider the total synthesis and the rate of synthesis as well as the specific activity of the enzymes.

The data presented show that the total quantity of either folate or riboflavin synthesized by *S. epidermidis* changes in response to physiological changes. The concentration of folic acid-like compounds varies

from about 15 ng/ml (0.008 nmole/mg dry weight) for organisms grown in the biotin-supplemented medium to about 500 ng/ml (2.2 nmoles/mg dry weight) for organisms grown in the synthetic medium. In addition to changes in nutrition, if the aeration is also changed, the concentration of folates synthesized ranges from less than 0.1 ng/ml to about 8000 ng/ml. The concentration of total riboflavin synthesized usually ranges from about 5 ng/ml (0.01 nmole/mg dry weight) for organisms grown in the biotin-supplemented medium to about 2500 ng/ml (3.7 nmoles/mg dry weight) for organisms grown in the FAAM.

Even though the total synthesis varies considerably both during growth and in different physical and nutritional environments, the intracellular concentrations of both folate and riboflavin always remain constant during exponential growth. Although remaining constant during growth, the intracellular concentrations do change with the nutritional composition of the medium. The intracellular concentration of folates varies from 0.0009 nmole/mg dry weight for organisms grown in the biotin-supplemented medium to 0.012 nmole/mg dry weight for organisms grown in the synthetic medium. The intracellular concentration of riboflavin varies from 0.018 nmole/mg dry weight in the biotin-supplemented medium to 0.45 nmole/mg dry weight for organisms grown in the FAAM. Assuming that *S. epidermidis* has 40% protein/mg dry weight, then the intracellular concentration of riboflavin ranges from 0.045 μmole/g protein to 1.1 μmoles/g protein depending upon the growth medium, compared to a constant intracellular concentration of riboflavin of 0.27 μmole/g protein as reported for *E. coli* (125). The constant intracellular concentration of either folate or riboflavin probably represents the physiological requirements of *S. epidermidis*

needed to maintain the maximum growth rate in a specific medium. When the biosynthesis of either folate or riboflavin exceeds the quantity required for growth, the excess is excreted into the culture medium.

The excreted folates and riboflavin do not increase at a constant rate during growth. Instead, there appears to be a loss of the excreted folates during the exponential growth phase. The apparent loss of folates is deceptive. I believe this is nothing more than the formation of conjugated pteridines which have different dose responses for *S. faecalis*. It was shown that the types of folic acid-like compounds found in the spent culture medium shift in relative concentration to one another depending upon the phase of growth. Some types of folic acid-like compounds are found in the spent culture medium of only young cultures while others are found only in the stationary phase cultures. Most of these biologically active derivatives are not present in large enough quantities to determine their dose response for *S. faecalis*. This explanation is plausible since the dose responses for the oxidized and reduced pteroic and folic acids are different in *S. faecalis*. Thus, it is probable that there are still the same number of molecules of the basic conjugated pteridine but they have been altered to a less active form.

The compounds that stimulate either folate or riboflavin synthesis may participate in the synthesis of these vitamins. Some examples: pABA is coupled to pteridines to make pteroates; xanthine or inosine could be changed intracellularly to a precursor of both the folates and riboflavin; pyridoxine could participate in the purine ring-opening reaction by forming a Shiff's base; 2,4,5-triamino-6-hydroxypyrimidine and uracil are structurally similiar to the proposed pyrimidine moiety of the folate and

riboflavin intermediates; and citrate may function in the synthesis of the 4-carbon donor that forms the lumazine ring system in riboflavin synthesis. With the exception of the role of pABA, the other explanations are hypothetical. All the compounds which stimulate folate or riboflavin synthesis also inhibit the growth of the organism. The more that growth is inhibited, the greater the relative synthesis per mg dry weight. The reverse also seems to be true, that any change in the nutritional environment that stimulates growth of the organism also decreases the relative synthesis of both the folates and riboflavin. Thus, when biotin is added to the synthetic medium, the length of the lag period is reduced, the generation time is reduced, the maximum population is nearly triple that for the synthetic medium alone, the folate synthesis per mg dry weight is reduced 50-fold and the riboflavin synthesis per mg dry weight is reduced 10-fold. Biotin not only decreases folate synthesis and stimulates growth, but it also decreases the intracellular concentration of folates about 15-fold. It is possible that the biotin interaction in some way partially replaces the function of folic acid, therefore, it decreases the requirement for folate synthesis. However, the general phenomenon of good growth-low folate and riboflavin synthesis suggests that an inhibitor is produced in large quantities when the organism is growing rapidly, resulting in decreased folate and riboflavin synthesis.

The rate of synthesis during exponential growth of a culture can be calculated from the formula:

$$R_{vs} = \frac{[V]_2 - [V]_1}{M_2 - M_1} \times \mu,$$

where R_{vs} is the rate of vitamin synthesis, $[V]_2$ is the total vitamin

content of the culture (intracellular + excreted) at the end of the incubation period, $[V]_1$ is the total vitamin content at the beginning of the incbation period, M_2 is the bacterial mass at the end of the incubation, M_1 is the mass at the beginning of the incubation and μ is the specific growth rate (13). A negative value indicates that growth exceeds vitamin synthesis resulting in a decrease in the vitamin concentration per cell per unit time. Under all experimental conditions the rate of synthesis of either the folates or riboflavin have negative values during exponential growth. This indicates that the growth rate exceeds the rate of folate or riboflavin biosynthesis during exponential growth. This is in direct conflict with reports of a constant positive rate of flavin biosynthesis during exponential growth in *E. coli* (125) and in *C. quilliermodii* (95). There is a positive rate of synthesis during the lag phase and during the transition phases of accelerated and decelerated exponential growth. Neither Wilson and Pardee (125) nor Shavlovskii and Strugovshchikova (95) examined flavin synthesis in the lag or accelerated exponential phases of growth. Both groups of investigators resuspended exponentially-growing cultures into fresh medium and then examined the rate of synthesis.

When *S. epidermidis* is grown under different physiological stresses, the total synthesis and the rate of synthesis of the folates and riboflavin should be a reflection of the enzymatic activity of the respective pathways. Since the growth medium can be manipulated in a way to force *S. epidermidis* to synthesize different quantities of folates and riboflavin relative to each other, the specific activities of the enzymes from the pteridine, folate and riboflavin pathways should also change relative to one another. I wanted to compare the activity of an enzyme to the total

synthesis of either the folates or riboflavin; therefore, crude cell-free extracts were used as a source of the enzymes.

There are two limitations related to the use of crude extracts. First, the crude extracts contain substrates, intermediates, end products and potential inhibitors, all of which interfere with the enzyme assays. Usually these small molecules are removed by dialysis, but the crude extracts could not be dialyzed because 99% of the pteroate synthetase activity is lost by dialysis. And second, a change in the specific activity of an enzyme from different extracts could be due to either a change in the quantity of the enzyme (induction-repression) or a change in the activity of the enzyme (feedback inhibition). When comparing enzymes prepared from organisms grown in different culture media, this could be a real problem. The specific activities of GTP-cyclohydrolase, pteroate synthetase and riboflavin synthetase decrease about 16-, 29- and 2-fold respectively when the synthetic culture medium is supplemented with biotin. But, when the activity of an enzyme is compared during growth, the rate at which the specific activity decreases distinguishes between repression and feedback inhibition. With feedback inhibition the enzyme activity would decrease abruptly, whereas with repression the decrease would be slow as the enzymes are diluted with cell division. The loss of specific activity can be greater than predicted for dilution alone if the enzyme is not only repressed, but is also being degraded. During early exponential growth in the synthetic medium, the specific activities of GTP-cyclohydrolase, pteroate synthetase and riboflavin synthetase decrease about 15-, 8- and 8-fold respectively. Both pteroate synthetase and riboflavin synthetase lose about one half of their specific activity per generation which

indicates that the enzymes are being diluted. GTP-cyclohydrolase loses more than half of its activity during the second generation of early exponential growth in synthetic medium; however, the loss during the first generation (about 50% of the enzyme activity

During exponential growth, GTP-cyclohydrolase, pteroate synthetase and riboflavin synthetase are repressed. If some of the end products of folate metabolism (thymine, thymidine, serine, glycine and methionine) are added to the growing culture, the pteroate synthetase activity does not increase, suggesting that the end products of folate metabolism repress the folate pathway. The addition of these compounds has no effect upon riboflavin synthetase activity. GTP-cyclohydrolase activity is not required for the synthesis of the folate pteridines since the pteroate synthetase is repressed, yet, GTP-cyclohydrolase is only partially inhibited by the end products of folate metabolism. Apparently another product of GTP-cyclohydrolase is required, possibly for riboflavin synthesis. Biotin also inhibits pteroate synthetase, has little effect upon riboflavin synthetase and partially inhibits GTP-cyclohydrolase, again suggesting that GTP-cyclohydrolase functions in both folate and riboflavin biosyntheses. This hypothesis is strengthened by the fact that both dimethyl-8-ribityllumazine, an intermediate in riboflavin biosynthesis, and hydroxymethylpteridine, an intermediate in folate biosynthesis, decrease the specific activity of GTP-cyclohydrolase when added independently and in combination to the culture medium.

On the basis of growth, excretion, rates of synthesis and specific activities of the enzymes from the pteridine, folate and riboflavin pathways, I can only conclude that the enzymes of both folate and

riboflavin biosynthetic pathways are not constitutive. Both pathways appear to have an elaborate control mechanism. The only feature that is inconsistent with an elaborate control mechanism is that under all experimental conditions *S. epidermidis* overproduces and excretes both folates and riboflavin.

Precision of adjustment.

The excretion of a biosynthetic end product signifies that the control mechanisms in the pathway are not precise enough to prevent the compound from being overproduced (27, 74, 125). Therefore, one criterion for the precision with which a biosynthetic pathway is controlled is the extent of overproduction. Wilson and Pardee (125) suggest that the ratio of excreted product to intracellular product (E/I ratio) should be a measure of the weakness of a control mechanism. They report that the E/I ratio of flavin production in *E. coli* ranges from 0.8 to 8.0 compared to about 0.05 for the nucleic-acid bases. Yet the absolute quantity of flavins that are excreted is no greater than the absolute quantity of amino acids or nucleic-acid bases that are excreted into the culture medium. This raises an interesting question. For a biosynthetic pathway that is fully repressed and under the influence of a feedback inhibition mechanism, how many moles of product are formed, if any? I don't know of any reports in the literature that state that feedback inhibition is an absolute inhibition of an enzyme. Bacteria that have inducible biosynthetic pathways still synthesize the enzymes in the absence of an inducer. Khogness (48) reports that when β-galactosidase is completely repressed and there is an absence of inducer in the medium, *E. coli* forms about 20

active β-galactosidase molecules per cell. This is by far more enzyme molecules than McIlwain (63) postulates are present for enzymes of any vitamin biosynthetic pathway.

The E/I ratio for either the folates or riboflavin in *S. epidermidis* varies greatly depending upon the phase of growth or the nutritional supplementation. During the lag and accelerated exponential phases of growth the E/I ratio is several hundred. The lowest E/I ratio for the folates (about 2.1) occurs during the mid-exponential growth. For riboflavin, the lowest E/I ratio (about 0.5) occurs in the early stationary phase of growth. Thus, neither the folate nor the riboflavin pathway has a very precise regulation when examined on the basis of overproduction.

It is not surprising that *S. epidermidis* does not have a precise regulatory mechanism for the biosynthesis of folic acid-like compounds. *S. epidermidis*, like most organisms used to study the biosynthesis of vitamins, was originally selected because it excreted large quantities of folic acid-like compounds. Investigators have either consciously or unconsciously selected organisms which excrete large quantities of the vitamin in question because the enzymes for the specific pathway should be present in a higher quantity or in a more active form in such an organism. Therefore, the intermediate compounds as well as the end product should be produced in relatively high quantities and the biochemical pathway should be easier to elucidate. These same organisms, specifically *E. coli, L. plantarum and S. epidermidis* in which the folic acid pathway has been determined to various degrees, are now being studied in attempts to demonstrate the mechanism of regulation. Since all of these organisms overproduce folic acid-like compounds, it is unlikely that they

are subject to exact regulation. Therefore, feedback inhibition, if it exists in this type of organism, must play a minor role in the overall regulation of folate biosynthesis. I unsuccessfully tried to find either mutants or strains of *S. epidermidis* which would not excrete folic acid-like compounds thinking that such an organism would be subject to exact regulation. However, I found one strain of *B. subtilus* and three isolates of *Acinetobacter* which excreted no detectable folic acid-like compounds. None of these organisms was examined in detail.

I was unable to find any folate auxotrophs of *S. epidermidis*. No folate auxotrophs have been found in *E. coli* (G. M. Brown, Massachusetts Institute of Technology, Cambridge, personal communication) or in *L. plantarum* (T. Shiota, University of Alabama Medical Center, Birmingham, personal communication). It was simple and attractive to explain the lack of folate auxotrophic mutants on the basis that the organism did not have a transport mechanism to get folic acid into the cell, therefore, the mutation was lethal. It is clear that folic acid can enter the cell since radioactive folic acid was found inside *S. epidermidis* when grown in the presence of exogenous radioactive folic acid. It is possible that a mutation in the folate pathway may also alter the permeability for folic acid, but currently there is no experimental evidence to support this hypothesis. Without folate auxotrophic mutants it was impossible to get double mutants requiring both folate and riboflavin for further biochemical and genetic studies.

If only the E/I ratio is considered, then *S. epidermidis* is not precisely regulated. However, the total quantity of excreted folates or riboflavin is no greater than the reported quantity of purine or

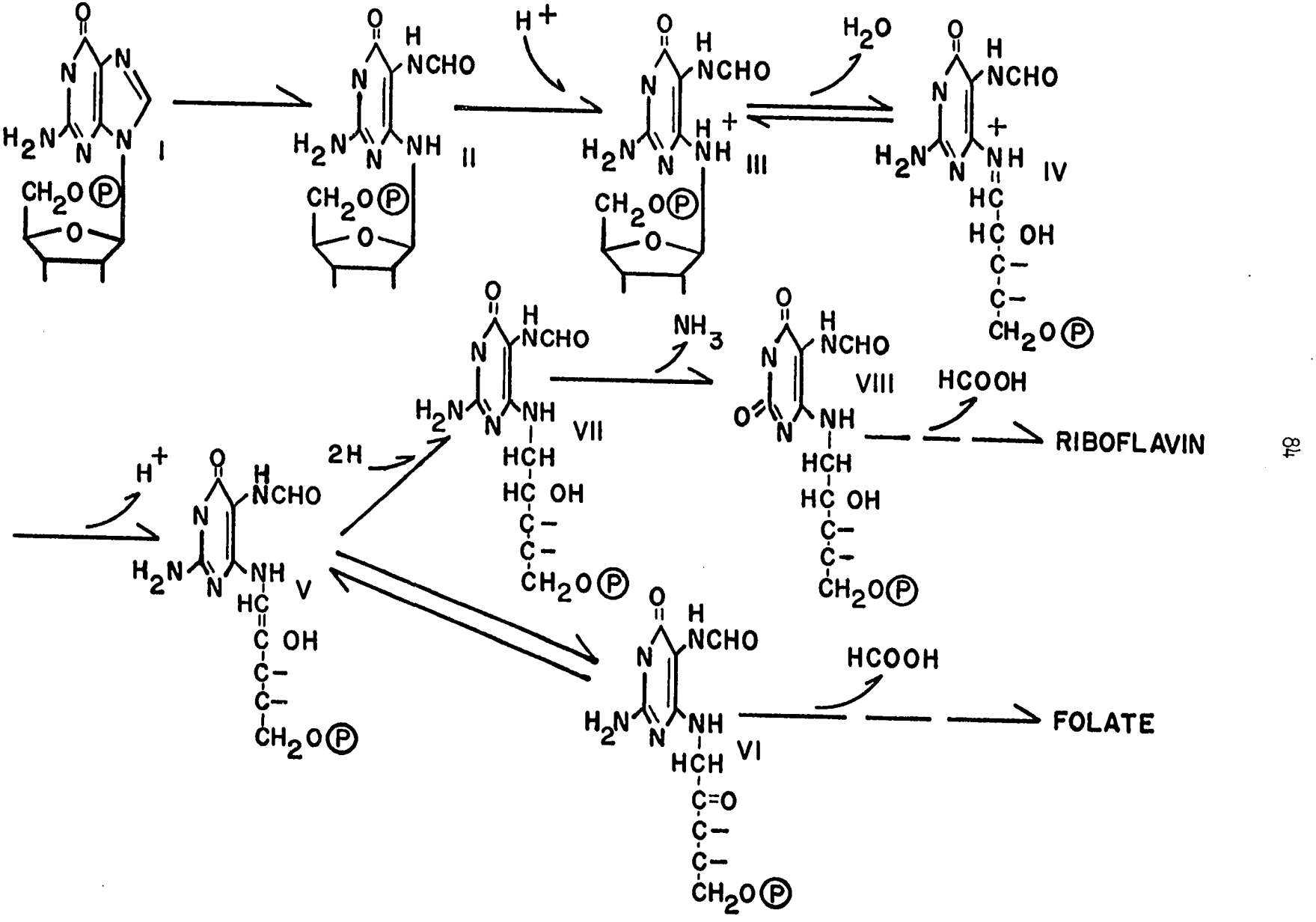
pyrimidines that are excreted. From this point of view, the regulatory mechanism for the folates or riboflavin is as precise as the regulatory mechanisms for the major biosynthetic pathways.

A branched pteridine biosynthetic pathway

Purines can serve as a precursor for both folate and riboflavin biosynthesis with the expulsion of C-8 of the purine (18, 23, 35, 56, 57, 58, 69, 70, 93, 102, 108). GTP has been established as the purine precursor of the folate pteridines (18, 23, 56, 57, 58, 102, 108) and guanine, or a nucleoside or nucleotide derivative of guanine, has been established as the purine precursor for riboflavin biosynthesis (4, 7, 35, 37). Both folic acid-like compounds and riboflavin are synthesized by *S. epidermidis*; thus, it is probable that the two pathways share a common precursor and have some common intermediates. GTP-cyclohydrolase is a large enzyme complex which catalyzes the elimination of C-8 of GTP in the synthesis of the folate pteridines and isomerizes the ribose moiety to a 1'-deoxy-2'-keto pentose unit by an Amadori rearrangement. I believe that some of the intermediates formed by the GTP-cyclohydrolase complex in the formation of the folate pteridines are also intermediates in riboflavin biosynthesis.

The Amadori rearrangement is an example of acid-base catalysis (36). It is necessary that the N-substituted ribosylamine be sufficiently basic to accept a proton. The Amadori rearrangement proceeds in three steps: 1) the acceptance of a proton by the ribosylamine base; 2) a prototrophic shift; and 3) the discharge of a proton from the cation originally formed. Fig. 9 shows the mechanism of the Amadori rearrangement and the hypo-

Fig. 9. A hypothetical scheme for a branched pteridine biosynthetic pathway.


I
II
III
IV
V
VI
VII
VIII
RIBOFLAVIN
FOLATE
HCOOH
HCOOH
NH3
2H
H+
H+
H2O

thetical branch point in the pteridine pathway. During the Amadori rearrangement, there is an initial attack by a proton on the nitrogen forming an ammonium ion (Compound III) which is in equilibrium with the cation of a Schiff's base (Compound IV). A shift of the double bond and a flow of electrons toward the positive nitrogen atom makes C-1' transiently positive. A second flow of electrons from C-2' to C-1' weakens the C-H bond on C-2', causing the expulsion of that proton to give the enol form (Compound V) of the ketose derivative (Compound VI). The tautomeric shift to the keto form (Compound VI) is driven by the tendency of Compound VI to form a stable ring.

Primary amines readily react with a carbonyl group while secondary amines react slowly or not at all if sterically hindered. Based upon the reactivity of primary amines and secondary amines with carbonyl groups, I propose that the initial enzymatic hydrolysis of the imidazole ring of GTP must cleave the ring between C-8 and N-9 of GTP leaving a formyl group attached at what was formerly N-7 of GTP, resulting in the formation of 2-amino-4-(1'-D-ribosylaminotriphosphate)-5-formamido-6-hydroxypyrimidine (Compound II). Shiota (103) has shown the 5-formamidopyrimidine structure to be correct and a stable derivative. If the initial hydrolysis was between N-7 and C-8 of GTP, then what was formerly N-7 of the purine would become a primary amine and would readily react with the formyl group to make GTP.

I propose that compound V is the branch point in the pteridine pathway and this compound leads to the synthesis of both folic acid-like compounds and riboflavin. A reduction of compound V would result in the formation of a 2-amino-4-(1'-D-ribitylamino)-5-formamido-6-hydroxypyrimi-

dine (Compound VII) or a phosphate ester thereof which can be oxidatively deaminated at the C-2 position of the pyrimidine forming a 4-(1'-D-ribityl-amino)-5-formamido-2,6-dihydroxypyrimidine derivative (Compound VIII). Both 2,5-diamino-4(1'-D-ribitylamino)-6-hydroxypyrimidine (3, 5, 6, 76) and 4-(1'-D-ribitylamino)-5-amino-2,6-dihydroxypyrimidine have been shown to be accumulated by riboflavin mutants of *S. cerivisiae* and *A. nidulans*.

The reason that I believe that the intermediates retain the original C-8 of GTP as a formyl group is: first, the electron withdrawing properties of the formyl group attached to the nitrogen would prevent a nucleophillic attack upon a carbonyl group of the Amadori rearrangement products until after the first true riboflavin intermediate was formed; and second, the pyrimidine-like intermediates formed from GTP-8-^{14}C are radioactive. The radioactive carbon is acid labile and is released as formic acid. One of these intermediates can be condensed with diacetyl yielding radioactive formic acid and a pteridine compound which resembles 6,7-dimethyl-8-ribityl-lumazine. Another radioactive intermediate forms a 6,7-dimethylpterin derivative when condensed with diacetyl.

Other supporting evidence for a branched pteridine biosynthetic pathway is: first, cultures grown in the presence of 6,7-dimethyl-8-ribityl-lumazine have a very low GTP-cyclohydrolase activity compared to cultures grown in the absence of 6,7-dimethyl-8-ribityllumazine; second, the GTP-cyclohydrolase activity is partially restored when 6,7-dimethyl-8-ribityl-lumazine is removed; and finally, GTP-cyclohydrolase activity changes with the same relative proportion as riboflavin synthetase activity and with no change in the pteroate synthetase activity.

Regulation of folate and riboflavin biosynthesis

Pardee and Beckwith (82) point out that the rate of synthesis of some repressed enzymes is hundreds or thousands of times greater than the rate of synthesis of other repressed enzymes. Yet, several investigators (63, 81, 82, 95, 125) have emphasized the low rate of vitamin and coenzyme synthesis compared to the rate of synthesis of the major metabolites. They have asked what prevents vitamins from being synthesized at the same rate as amino acids, purines or pyrimidines, only to conclude that there must be one or a very few molecules of the vitamin biosynthetic enzymes per cell. These same investigators have not questioned the mechanism which prevents the major metabolic pathways from functioning at the same rate as the catabolic pathways for a primary carbon source. This is the same question! These two mechanisms must be similar, simple, able to withstand evolutionary selection and in essence a function of the genetic code.

The regulation of the enzymes of the catabolic and the major metabolic pathways is defined in terms of constitutivity, induction-repression and negative feedback inhibition. The constitutive enzymes are produced at a relatively constant rate independent of nutritional conditions. In contrast to the constitutive enzymes, the enzymes subject to induction-repression are synthesized at different rates depending upon the nutritional conditions. Finally, negative feedback inhibition controls the activity of enzymes already synthesized.

The adjustment of folate and riboflavin biosynthesis in response to nutritional changes can be explained by induction-repression. With the exception of overproduction, the criteria for an induction-repression

mechanism have been demonstrated. The riboflavin synthetase enzyme appears to be constitutive based upon the negligible change in the specific activity in response to changes in nutrition. But the change in total riboflavin synthesis in response to nutritional changes indicate that the pathway is subject to induction-repression.

It is difficult to evaluate the role of negative feedback inhibition, especially since GTP-cyclohydrolase and pteroate synthetase are enzyme complexes which catalyze more that one reaction. I believe that a weak, sequential feedback mechanism does function in the folate biosynthetic pathway. It appears as if the products of folate metabolism do feedback inhibit the pteroate synthetase. I suspect that the purine ring-opened intermediate feedback inhibits the initial hydrolytic reaction in the GTP-cyclohydrolase complex. Based upon overproduction and excretion of folates and riboflavin, feedback inhibition does not play a significant regulatory role in the biosynthesis of folates and riboflavin in *S. epidermidis*. The lack of a precise regulatory mechanism should be expected in *S. epidermidis* because the organism was originally selected on the basis of the large quantities of folic acid-like compounds which it excretes.

SUMMARY

Folate and riboflavin biosyntheses appear to be regulated by an induction-repression mechanism. There is rapid synthesis of both vitamins during lag and accelerated exponential growth; synthesis stops or is severly reduced during exponential growth; and synthesis is again initiated during decelerated exponential growth. Both folate and riboflavin biosyntheses are reduced when the culture medium is supplemented with biotin; and the syntheses of both are partially restored when the biotin is then removed from the culture medium. Purines, pyrimidines, citrate and pyridoxine stimulate folate and riboflavin biosyntheses, but all these compounds also inhibit growth of *S. epidermidis*. Under all experimental conditions, *S. epidermidis* overproduces and excretes both folates and riboflavin.

The activity of GTP-cyclohydrolase is inhibited when *S. epidermidis* is grown in the presence of 6,7-dimethyl-8-ribityllumazine, hydroxymethyl-pteridine or biotin; the activity is partially restored when these compounds are subsequently removed from the culture medium. Pteroate synthetase is inhibited when the products of folate metabolism (thymine, thymidine, serine, glycine and methionine) are supplemented into the culture medium. Riboflavin synthetase appears to be constitutive.

GTP-cyclohydrolase seems to function in the syntheses of both folate and riboflavin pteridines, suggestion a branched pteridine pathway.

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APPENDIX

Table A1. The morphological and biochemical characteristics of *S. epidermidis*.

Cellular Morphology	Spherical to ovoid cells, 0.5 to 1.5 microns in diameter. Non-motile. Occurring in singles, pairs, tetrads and clusters. Gram positive.
Colony Morphology Tripticase Soy Agar	Convex, glistening, entire margin, 1 mm in diameter.
Pigmentation	White. Becomes browish-yellow within 48 hr when exposed to light at room temperature.
Growth	
Nutrient Broth	Heavy growth. At first turbid, later clear with mucoid sidiment.
BHI Slant	Heavy growth, white, smooth, glistening, viscous.
Staph 110	Positive.
10% NaCl	Positive.
Litmus Milk	Acid in 24 hr. Coagulated in 48 hr.
Oxygen Requirement	Facultative anaerobe.
Biochemical	
Starch Hydrolysis	Negative.
Gelatin Hydrolysis	Positive in 48 hr.
Hemolysis	α on rabbit blood agar.
Catalase	Positive.
Coagulase	Negative.
Urease	Positive.
Nitrate	Reduced.
Indol	Negative.
Lysis	Sensitive to lysostaphin. Resistant to lysozyme

Table A1. Continued.

Sugar Utilization	Fermentative. Acid from glucose, lactose, sucrose. No growth on Mannitol, maltose, inulin, raffinose, sorbitol, trehalose.
Growth Requirements	
Purines	None.
Pyrimidines	None.
Vitamins	Niacin. Niacin and thiamine are required when pyruvate is the primary carbon source.
Amino Acids	Many. Growth in the absence of glycine, serine, alanine, methionine, histidine, tyrosine, phenylalanine.
DNA Composition	31 mole per cent G + C (buoyant density).
Temperature	Slight growth at 10 C. Moderate growth at 45 C. Optimum at 35 C.

Fig. A1. The relationship of optical density to dry weight. Corrected optical density for growth in synthetic medium (□); corrected optical density for growth in FAAM (○); Uncorrected optical density for growth in FAAM (△); optical density for growth in FAAM, 1:5 dilution after OD=1.0 (▲) [see Growth determinations, EXPERIMENTAL PROCEDURE].

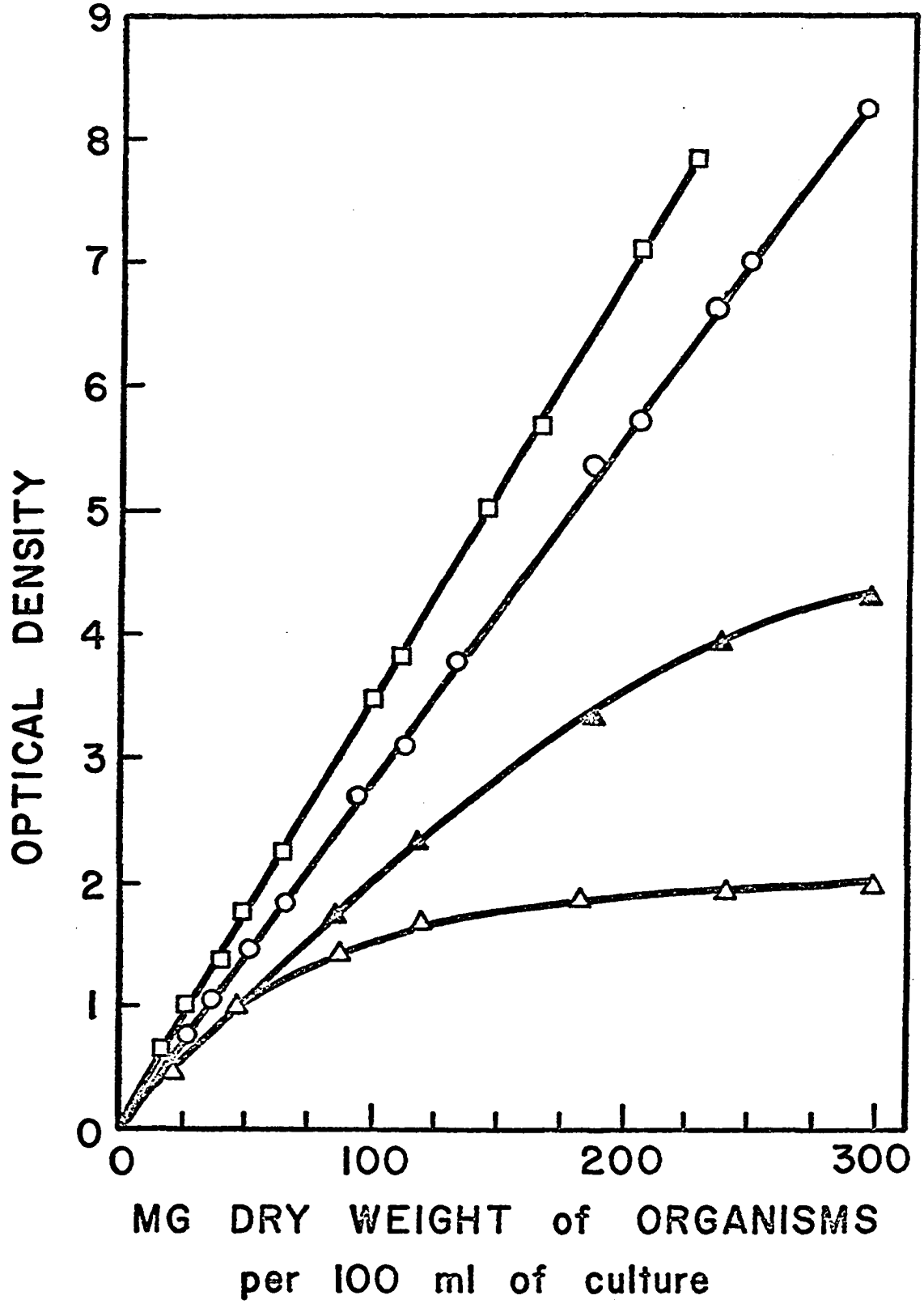
OPTICAL DENSITY
0
1
2
3
4
5
6
7
8
9
0
100
200
300
MG DRY WEIGHT of ORGANISMS
per 100 ml of culture

Table A2. R_f values and biological activity for standard compounds.

Compound	Solvent System A	B	C	Biological Activity *S. faecalis*	*L. casei*
GTP	0.93	0.01	0.16	0[a]	0
GDP	0.89	0.05	-[b]	0	0
GMP	0.85	0.11	-	0	0
l-Neopterin	0.68	0.18	-	0	0
d-Neopterin	0.67	0.20	-	0	0
Pterin	0.58	0.37	0.48	0	0
6,7-dimethylpterin	0.57	0.46	0.62	0	0
Pteridine-6-COOH	0.51	0.15	0.23	0	0
Pteridine-6-CH_2OH	0.58	0.27	0.40	0	0
Xanthopterin	0.39	0.32	0.29	0	0
Isoxanthopterin	0.45	0.22	0.30	0	0
Lumazine	-	0.41	0.50	0	0
6,7-dimethyl-8-ribityl-lumazine	0.74	0.21	0.49	0	0
Riboflavin	0.36	0.54	0.53	0	+++
FMN	0.56	0.14	0.25	0	+++
FAD	0.45	0.06	-	0	+++
Folate	0.35	-	-	+++	+++
Dihydrofolate	0.17	-	-	+++	+++
N^{10}-formylfolate	-	-	-	+++	+++
Pteroate	0.01	-	-	+++	0
Rhizopterin	0.62	0.56	-	+++	0
Pteroyl-γ-triglutamate	-	-	-	0	+++
Pteroyl-γ-heptaglutamate	-	-	-	0	0

[a]Not biologically active.

[b]Not determined.

Fig. A2. The elution profile from Celite 535 showing radioactive formic acid releases from GTP-8-^{14}C (0.3 mM, 5.2 X 10^5 DPM) by GTP-cyclohydrolase from different preparations: crude extracts (●); heat-treated crude extracts (○); partially purified extracts, Sephadex G-75, Peak I (□); partially purified extracts, Sephadex G-75, Peak II or Peak III (△). The inset is the elution profile of authentic ^{14}C-formic acid [see Purification of GTP-cyclohydrolase, EXPERIMENTAL PROCEDURE].

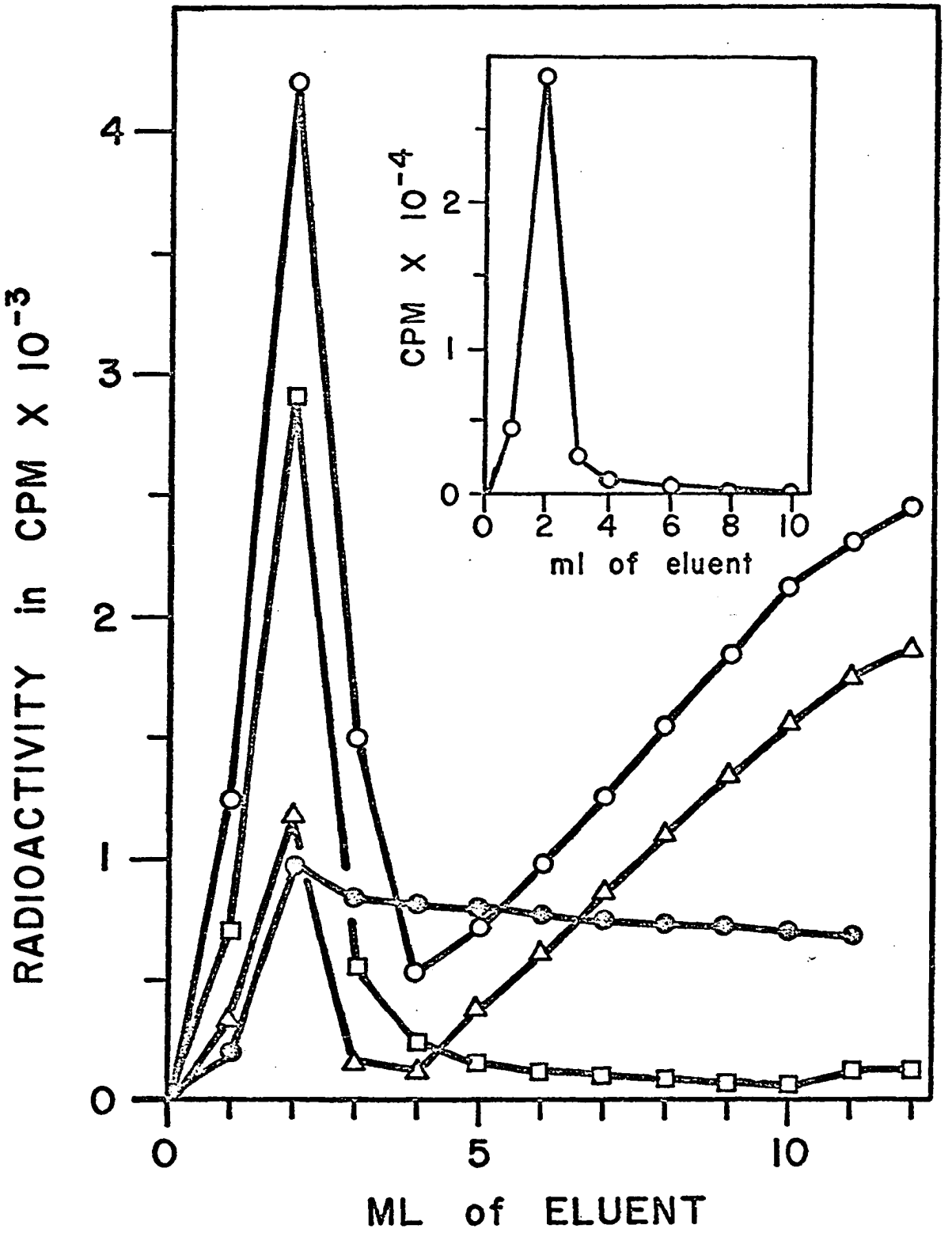
RADIOACTIVITY in CPM X 10⁻³
ML of ELUENT
0
5
10
0
1
2
3
4
CPM X 10⁻⁴
ml of eluent
0
2
4
6
8
10

Fig. A3. Growth curves in synthetic medium for *S. epidermidis* strain W, mutant NFA, mutant NFB and eight strains from NADL: A-2; A-5; A-110; A-197; A-241; A-283; A-285; and A-286.

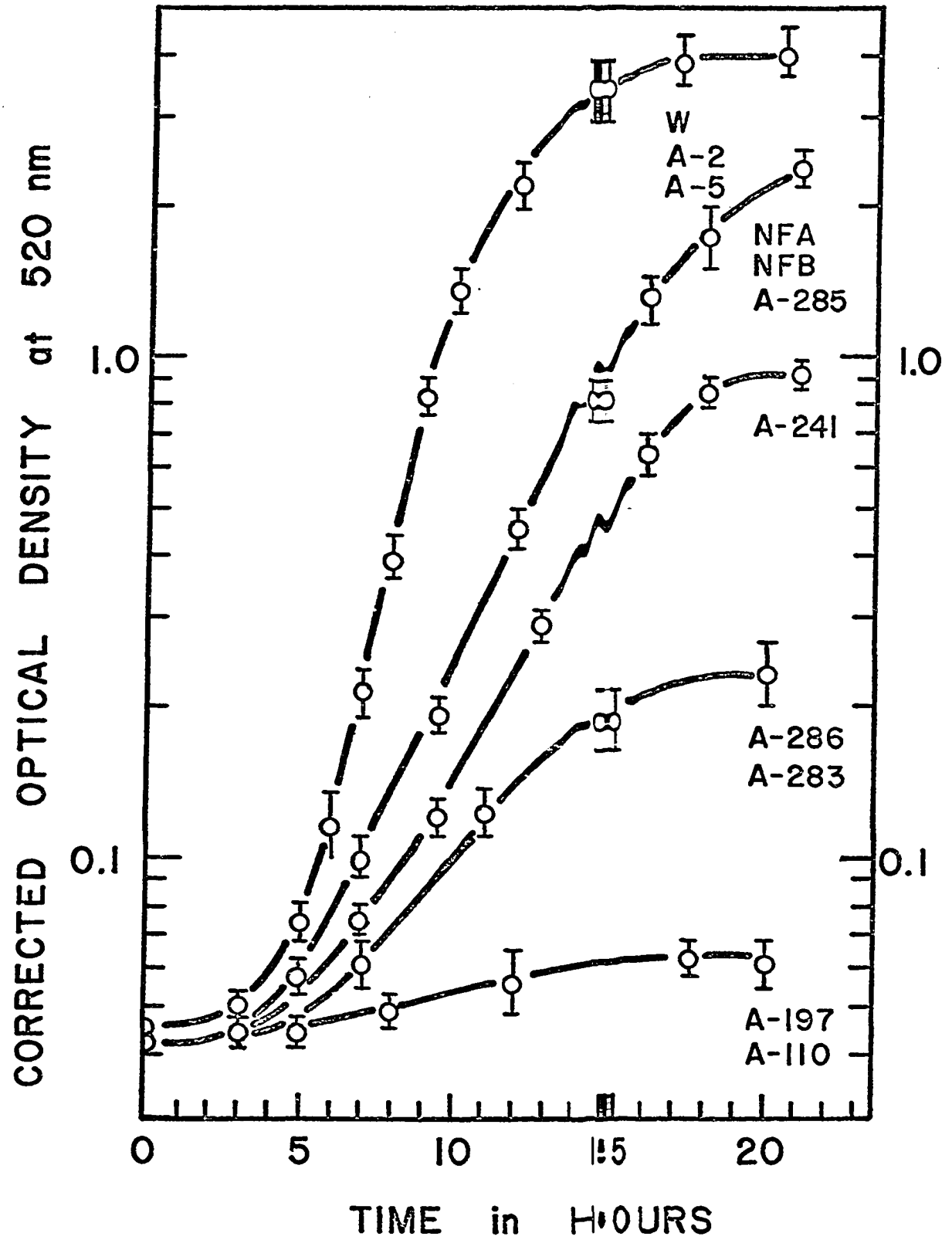
CORRECTED OPTICAL DENSITY at 520 nm
1.0
0.1
0
5
10
15
20
TIME in HOURS
W
A-2
A-5
NFA
NFB
A-285
A-241
A-286
A-283
A-197
A-110

Table A3. A comparison of growth and folate excretion in stationary and shake flasks by strains of *S. epidermidis* grown in synthetic medium.

Strain	Aerated		Non-aerated	
	Growth OD_c	Folate equivalents ng/ml	Growth OD_c	Folate equivalents ng/ml
W	3.0	210	0.72	26
A-2	3.4	185	0.89	43
A-5	3.2	194	0.85	50
NFA	2.6	136	0.80	48
NFB	2.5	120	0.59	44
A-285	2.6	125	0.45	2
A-241	1.0	64	0.84	45
A-286	0.3	34	0.33	4
A-283	0.2	17	0.19	5

Table A4. The R_f values in Solvent A of the excreted folates by strains of *S. epidermidis* grown in synthetic medium.

Strain	R_f Values				
	0.01	0.11	0.25	0.45	0.57
W	+	+	-	+++	++++
A-2	+	+++	-	++	++++
A-5	+	++	-	++	++++
NFA	+	++	-	+++	++++
NFB	+	++	+++	++	++++
A-285	++	±	±	±	±
A-241	++	±	-	±	±
A-286	+	-	-	-	-
A-283	±	-	-	-	-